UNIVERSIDAD COMPLUTENSE DE MADRID

FACULTAD DE CIENCIAS BIOLÓGICAS

DEPARTAMENTO DE FISIOLOGÍA ANIMAL (FISIOLOGÍA II)

TESIS DOCTORAL

Relevancia de la ruta de la anemia de Fanconi en la inestabilidad genética de las células de leucemia mieloide crónica

MEMORIA PARA OPTAR AL GRADO DE DOCTOR

PRESENTADA POR

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Memoria presentada por **Antonio Valeri Lozano** para optar al grado de Doctor por la Universidad Complutense de Madrid.

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El **Dr. Juan Antonio Bueren Roncero**, Jefe de la División de Terapias Innovadoras en el Sistema Hematopoyético del Centro de Investigaciones Energéticas, Medioambientales y Tecnológicas (CIEMAT) y la **Dra. Paula Río Galdo**, Científico titular en la División de Terapias Innovadoras en el Sistema Hematopoyético del Centro de Investigaciones Energéticas, Medioambientales y Tecnológicas (CIEMAT) certifican que la memoria adjunta titulada "RELEVANCIA DE LA RUTA DE LA ANEMIA DE FANCONI EN LA INESTABILIDAD GENÉTICA DE LAS CÉLULAS DE LEUCEMIA MIELOIDE CRÓNICA" ha sido realizada por el licenciado Don Antonio Valeri Lozano, bajo la dirección de los que suscriben, y cumple con las condiciones exigidas para optar al título de doctor por la Universidad Complutense de Madrid.

El trabajo de investigación descrito en esta memoria ha sido realizado en la División de Terapias Innovadoras en el Sistema Hematopoyético del Centro de Investigaciones Energéticas Medioambientales y Tecnológicas (CIEMAT) y del Centro de Investigación Biomédica en Red de Enfermedades Raras (CIBERER) y la Unidad Mixta de Terapias Avanzadas CIEMAT/ Instituto de Investigación Sanitaria Fundación Jiménez Díaz (IIS-FJD, UAM).

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ÍNDICE

ABREVIATURAS

ADN	Ácido desoxirribonucleico
AF	Anemia de Fanconi
AF/BRCA	Ruta de Anemia de Fanconi/ susceptiblidad a cáncer de mama
AKT	Proteína cinasa serina-treonina específica. Del inglés, *v-akt murine thymoma viral oncogene homolog 1*
AntagomiR	Oligonucleótidos modificados químicamente que inhiben la expresión de miARNs
Anti-miR	Inhibidores específicos de miARNs
ARE	Elementos de respuesta antioxidante
ARN	Ácido ribonucleico
ARNm	Ácido ribonucleico mensajero
ATM	Proteína mutada de la Ataxia telangiectasia. Del inglés *Ataxia Telangiectasia Mutated*
ATR	Proteína relacionada con RAD3 y de la Ataxia telangiectasia.
BAD	Del inglés *Bcl-2-Associated Death promoter protein*
BASC	Complejo de vigilancia genómica asociado a BRCA1
BCL-2	Del inglés *B-Cell Lymphoma 2*
BCR	Del inglés *Breakpoint Cluster Region*
BCR-ABL1	Oncogén de fusión
BER	Reparación por escisión de base. Del inglés *Base Excision Repair*
BLM	Proteína de Bloom
b-NHEJ	Del inglés *back-up Non Homologous End-Joining*
BRCA1	Gen supresor de tumores humano. Del inglés *Breast cancer 1, early onset*
c-ABL1	Del inglés *Abelson murine Leukemia viral oncogene homolog 1*
CB-LMC	Fase de crisis blástica de la leucemia mieloide crónica
CCE	Cáncer de células escamosas
CEBPα	Del inglés *CCAAT/enhancer-binding protein alpha*
CECC	Cáncer escamoso de cabeza y cuello
CMH	Célula madre hematopoyética
CML	Célula madre leucémica
Complejo ID	Complejo formado por las proteínas FANCD2 y FANCI
CPNM	Cáncer de pulmón no microcítico
CtIP	Del inglés *CtBP-interacting protein*
DDR	Respuesta al daño en el ADN. Del inglés *DNA Damage Response*
DEB	Diepoxibutano
DGCR8	Del inglés *DiGeorge syndrome Critical Region protein 8*

DNA-PK	Proteina cinasa dependiente de ADN. Del inglés *DNA-Dependent Protein Kinase*
DNA-PKc	Subunidad catalítica de proteína cinasa dependiente de ADN DNA-PK.
DSB	Rotura de doble hebra. Del inglés *Double Strand Break*
EGR1	Del inglés Early growth response protein 1
FAAP100	Proteína asociada a Fanconi de 100 KDa
FAAP20	Proteína asociada a Fanconi de 20 KDa
FAAP24	Proteína asociada a Fanconi de 24 KDa
FA-LMC	Fase acelerada de la leucemia mieloide crónica
FANCD2	Proteína D2 de la Anemia de Fanconi
FANCD2-S	Forma no ubiquitinada de FANCD2. Del inglés *FANCD2- Short*
FANCD2-Ub / FANCD2-L	Forma monoubiquitinada de FANCD2. Del inglés *FANCD2-Long*
FC-LMC	Fase crónica de la leucemia mieloide crónica
FDA	Del inglés *Food and Drug Administration*
FOXO3a	Factor de transcripción. Del inglés *Forkhead box O3*
H2AX	Del inglés *H2A histone family, member X*
HCV	Virus de la Hepatitis C
hnRNP-E2	Del inglés *heterogenous nuclear ribonucleoprotein E2*
Homeo RR	Reparación por recombinación homóloga no fidedigna
HR	Mecanismo de reparación de recombinación Homóloga. Del inglés *Homologous Recombination*
ICL	Enlace intercatenario, del inglés *Interstrand Cross Linker*
Indels	Inserciones y deleciones.
LLA-B	Leucemia linfoblástica aguda tipo B
LMA	Leucemia mieloide aguda
LMC	Leucemia mieloide crónica
LMC-N	Leucemia neutrofílica crónica
LNA-AntimiR	Inhibidores específicos de miARNs, del inglés *Locked Nucleic Acid- AntimiR*
LV	Vector lentiviral
LY294002	Inhibidor de PI3K
MAPK	Del inglés *Mitogen Activated Protein Kinase*
M-bcr	Punto mayor de ruptura. Del inglés *Major breakpoint region*
m-bcr	Punto menor de ruptura. Del inglés *minor breakpoint region*

MG132	Inhibidor del proteosoma
miARN	ARN monocatenario de pequeño tamaño capaz de regular la expresión de otros genes
MMC	Mitomicina C
MMR	Mecanismo de reparación de desapareamiento de bases. Del inglés *Mismatch Repair*
MNC	Células mononucleadas
MNR	Complejo formado por MRE11-RAD50-NBS1
mTOR	La diana de rapamicina en células de mamífero. Del inglés m*ammalian Target of Rapamycin*
NAC	N-Acetil cisteína
NER	Mecanismo de reparación de escisión de nucleótidos. Del inglés *Nuleotide Excision Repair*
NHEJ	Mecanismo de reparación de unión de extremos no homólogos. Del inglés *Non Homologous End-Joining*
NRF2	Del inglés *Nuclear factor erythroid 2–Related Factor 2*
OIS	Senecencia inducida por el oncogén. Del inglés *Oncogene Induced Senescence*
OncomiR	miARN que actúa como oncogén
p53	Del inglés *Tumor Protein p53*
PARP	Poli-ADP-Ribosa Polimerasa. Del inglés *Poly (ADP-Ribose) Polymerase*
pb	Par de bases
PGM	progenitores granulo-macrofágicos
Ph	Filadelfia. Del inglés *Philadelphia*
PI3K	Fosfatidil-inositol-cinasa-3. Del inglés, *Phosphatidylinositol 3-Kinase*
PICS	Senescencia inducida por pérdida de *PTEN*. Del inglés *PTEN-loss Induced Cell Senescence*
PML	Leucemia promielocítica
PP2A	Serina-treonina fosfatasa 2A. Del inglés *Protein Phosphatase 2A*
Pre-miARN	Precursor del miARN procesado y con un tamaño aproximado de 60-100 nucleótidos
Pri-miARN	Tránscrito primario del miARN con CAP en 5' y polyA en 3'.
PTEN	Fosfatasa y homólogo de tensina. Del inglés *Phosphatase and Tensin Homolog*
qPCR	Reacción en cadena de la polimerasa cuantitativa
RFP	Rotura-fusión-puente
RISC	Complejo inductor del silenciamiento. Del inglés RNA-induced silencing complex
ROS	Especies reactivas de oxígeno. Del inglés *Reactive Oxigen Species*
RV	Vector retroviral

SAHF	Del inglés *Senescence-Associated Heterochromatin Foci*
SCU	Sangre de Cordón umbilical
shRNA	Del inglés *short hairpin RNA*
SIPS	Senescencia prematura inducida por estrés. Del inglés *Stress Induced Premature Senescence*
SOD2	Superóxido dismutasa 2
SSA	Reparación por alineamiento de cadena simple. Del inglés *Single Strand Annealing*
SSB	Del inglés *Single Strand Break*
ssDNA	ADN de cadena única. Del inglés *Single Strand DNA*
STAT5	Del inglés *Signal Transducer and Activator of Transcription 5*
TKI	Inhibidores de tirosina cinasas
TLS	Síntesis a través de la lesión. Del inglés *Translation Synthesis*
UTR	Región no traducida del gen. Del inglés *Untranslated Region*
UV	Radiación ultravioleta
v-ABL	Gen transformante del virus de la leucemia murina de Abelson
WRN	Proteína de Werner
γH2AX	Forma fosforilada de H2AX en la serina 139
53BP1	Proteína 1 de unión a p53. Del inglés P*53-Binding Protein 1*
8-oxodG	8-Oxo-desoxiguanina

I. SUMMARY

RELEVANCE OF THE FANCONI ANEMIA PATHWAY IN THE GENETIC INSTABILITY OF CHRONIC MYELOID LEUKEMIA.

1.Introduction

Chronic Myeloid Leukemia (CML) is a clonal hematopoietic disorder generated by the translocation t(9;22)(q34;q11), resulting in the *BCR-ABL1* oncogene that encodes for a tyrosine kinase BCR-ABL1 oncoprotein. Although several genetic defects accumulate in CML cells during the progression from the chronic phase towards the accelerated and blast crisis phases, the generation of CML in mice transplanted with bone marrow cells carrying the *BCR-ABL1* fusion demonstrated that this oncogene is the causative agent of CML.

In addition to a differentiation arrest, failures in the genomic surveillance and DNA repair of CML cells account for the natural malignant progression of the disease. Although the mechanisms by which BCR-ABL1 interferes with the genomic stability of the cell are still poorly understood, the progression to blast crisis does not seem to depend on the acquisition of specific mutations in a single critical gene, but rather reflects a general state of genomic instability. Moreover, increasing evidence has been published showing that BCR-ABL1 induces reactive oxygen species (ROS) causing oxidative damage to CML cells. This effect results in a variety of DNA lesions, including the highly mutagenic double strand breaks (DSBs).

Regarding the mechanisms by which BCR-ABL1 affects DNA repair, previous studies have shown that this oncoprotein interferes both with the non-homologous end joining (NHEJ) pathway and with other pathways that utilize homologous templates. In the context of NHEJ, it has been suggested that BCR-ABL1 could increase both classic and back up NHEJ activity, reducing the DNA repair fidelity. In addition to NHEJ, BCR-ABL1 has also been involved in the aberrant regulation of the two pathways that utilize homologous templates, the faithful homology directed repair (HDR) and the mutagenic single strand annealing (SSA). Interestingly, previous studies have shown that BRCA1, a critical protein for preserving the genomic integrity by promoting homologous recombination, is nearly undetectable in CML cells. Additionally, more recent studies have shown that BCR-ABL1 specifically promotes the repair of DSBs through SSA, a mutagenic pathway that involve sequence repeats.

According to the current state of the art it is generally accepted that the Fanconi anemia (FA) pathway coordinates several DNA repair pathways and also has an important role in ROS detoxification, being considered an essential pathway to maintain the genomic stability of the cell.

Sixteen different FA proteins have been identified in the FA pathway, each of them participating in one of the three FA protein complexes: The upstream complex – the FA core complex - is integrated by eight FA proteins (FANCA, FANCB, FANCC, FANCE, FANCF, FANCG, FANCL, FANCM) and three FA associated proteins (FAAP20, FAAP24 and FAAP100). A second complex is formed by FANCD2 and FANCI, which work together in the FA-ID complex. Finally, the monoubiquitinated FANCD2-FANCI proteins interact with downstream FA proteins such as FANCJ/BRIP1, FANCN/PALB2 and FANCD1/BRCA2 and RAD51C/FANCO which form stable complexes with proteins participating in HDR, like BRCA1 and RAD51. Nucleases such as FANCQ/XPF and FANCP/SLX4 (scaffold for other nucleases), responsible for the unhooking of the DNA, have been also recently described as members of FA pathway.

2. Objectives

Based on the above-mentioned observations and given that no studies had investigated the role of the FA pathway in the genomic instability of BCR-ABL1 cells, the main purpose of this work was to clarify the role of FA pathway in the characteristic chromosomal instability observed in CML.

To achieve this objective, the specific aims of the present work were the following:

• To study the role of FA/BRCA pathway in the genetic instability in CML cells.

• To unravel the molecular mechanisms disrupting the FA/BRCA pathway in BCR-ABL1 cells.

3. Results

The results presented in this study demonstrate for the first time that CML cells show a defect in a central FA pathway protein, FANCD2. Although FANCD2 was monoubiquinated in these cells, FANCD2 foci formation was markedly reduced. This result was confirmed not only in $CD34^+$ cells obtained from CML patients, but also in healthy donor umbilical cord blood $CD34^+$ cells transduced with *BCR-ABL1*-retroviral vectors (RVs), demonstrating that the BCR-ABL1 oncoprotein - and not secondary events generated during the progression of the disease - is responsible for the impaired FANCD2 foci formation in CML cells. Additionally, our results showed that the ectopic expression of BCR-ABL1 in $CD34^+$ cells also induced chromosomal instability in these cells, characterized by aberrant centrosomes and chromosomal aberrations.

Since FANCD2 mobilization to DNA repair foci is dependent on the presence of BRCA1 in the cell, and taking into account that BRCA1 protein levels are reduced in

CML cells, we demonstrated that the re-expression of BRCA1 in BCR-ABL1 $CD34^+$ cells restores FANCD2 foci in these cells. Even more, the ectopic expression of BRCA1 in BCR-ABL1 cells markedly reduced the number of chromosomal and centrosomal aberrations. This observation demonstrates for the first time that BRCA1 is responsible, at least in part, for the genetic instability in CML by restoring the formation of FANCD2 foci formation during S phase and diminishing centrosomal aberrations during the mitosis phase.

Although previous studies showed that BCR-ABL1 represses BRCA1 at the post-transcriptional level, the mechanisms involved in this down-regulation had not been clearly elucidated. Our study showed that the inhibition of either PI3K/AKT or the activity of the proteasome can partially restore the generation of BRCA1 and FANCD2 foci in BCR-ABL1 progenitor cells after MMC treatment, suggesting that BRCA1 can be regulated by an ubiquitin-degradation mechanism in CML cells.

Taking into account that microRNAs (miRNAs) are involved in the development and progression of various types of human cancers, and considering that they also constitute important regulators of healthy and malignant hematopoiesis, we also hypothesized that miRNAs could play an important role in the BRCA1 down-regulation observed in BCR-ABL1 cells. Combining *in silico* analysis and previous studies in CML patients, we investigated the role of miR-183-96-182 cluster in the expression of BRCA1 in CML cells. Our results showed that BCR-ABL1 down-regulates BRCA1 by increasing the expression of the miR-183-96-182 cluster; an observation that was verified both in CML progenitor cells and also in $CD34^+$ cells transduced with *BCR-ABL1*-RVs. The down-regulation of this cluster by lentiviral vectors expressing shRNA against the different members of the cluster not only increased the level of BRCA1 in BCR-ABL1 cells but also increased the expression of two key tumor suppressor genes: *FOXO3a* and *PTEN*. This increment also correlated with a marked decrease in ROS production and spontaneous chromosomal instability. Furthermore, the inhibition of miR-183-96-182 cluster also restored BRCA1 and FANCD2 functionality, allowing the mobilization of both proteins to DNA repair foci after DNA damage, thus reverting FA pathway alterations previously observed in CML cells. These results suggested that the BCR-ABL1 oncoprotein induces genetic instability in BCR-ABL1 progenitors by the de-regulation of miR-183-96-182 cluster.

Strikingly, the over-expression of the miR-183-96-182 cluster induced a significant reduction in the proliferation and clonogenic capacity of BCR-ABL1 hematopoietic progenitors, mainly due to an increase in apoptosis and senescence. We confirmed in these cells that the senescence process was triggered very rapidly in the absence of hyperproliferation and was associated with the expression of p21 and p53, and

correlated with the absence of HDM2, the p53 ubiquitin-ligase. Moreover, although DNA damage was induced in these cells, neither senescence associated heterochromatin foci nor activation of ATM was noted, recapitulating the so-called PTEN induced cell senescense (PICS). As far as we know, this is the first demonstration of a PICS phenotype in progenitor cells of the hematopoietic lineage, opening the possibility of inducing this kind of senescence to eliminate quiescent BCR-ABL1 cells, which are responsible for leukemia maintenance.

4. Conclusions

Taken together, the results presented in this study reveal for the first time the role of the FA/BRCA pathway in the genomic instability of CML progenitor cells. Our data also demonstrate that epigenetic mechanisms are responsible, at least in part, for the disruption of the FA pathway in CML cells. These studies point out unprecedented aspects of CML cells showing a link with the FA pathway, and may hopefully contribute to the design of new therapeutic approaches for the treatment of CML.

II. RESUMEN

La leucemia mieloide crónica (LMC) es una enfermedad neoplásica de la célula madre hematopoyética que se caracteriza por la expresión del oncogén de fusión *BCR-ABL1*. La inestabilidad genética que cursa durante la progresión de la LMC y que alcanza su máximo durante la crisis blástica (CB-LMC), ha sido objeto de numerosos estudios que han tratado de dilucidar cuales son los mecanismos por los que BCR-ABL1 genera daño en el ADN o altera la respuesta celular a ese daño en la LMC.

La ruta de la Anemia de Fanconi/susceptibilidad al cancér de mama (AF/BRCA) coordina varios mecanismos de reparación del ADN y permite, como funciones más importantes, eliminar el daño causado por los enlaces intercatenarios (ICL) en el ADN y participar tanto en la protección como en la recuperación de los bloqueos en las horquillas de replicación. Por tanto, la ruta de la AF/BRCA está implicada directamente en el control de la estabilidad genética de las células. Además, dado que se han detectado alteraciones genéticas y epigenéticas en esta ruta en cánceres esporádicos de distinta naturaleza, el objetivo principal de este trabajo ha sido el de investigar el papel de la ruta AF/BRCA en la inestabilidad genética de células de LMC.

Nuestros resultados han mostrado por primera vez que en células de pacientes con LMC se produce una deficiencia en la formación de focos de reparación de FANCD2, proteína central de la ruta AF/BRCA, tanto en células en división como tras el daño con un agente inductor de ICL. Este resultado se reprodujo utilizando células $CD34^+$ de sangre de cordón umbilical humano (SCU) transducidas con vectores retrovirales que expresan *BCR-ABL1*. En este modelo también se esclareció que la activación por monoubiquitinación de la proteína FANCD2 no estaba afectada como consecuencia de la expresión de *BCR-ABL1*. La expresión ectópica de *BCR-ABL1* en las células $CD34^+$ indujo la aparición de centrosomas supernumerarios y aberraciones cromosómicas y paralelamente, resistencia celular frente a agentes entrecruzantes del ADN. La expresión ectópica del supresor de tumores BRCA1 restauró la formación de focos de reparación de FANCD2 y redujo la aparición de cromosomas y centrosomas aberrantes en los progenitores hematopoyéticos *BCR-ABL1*, poniendo de manifiesto que la disminución de la expresión de BRCA1 en LMC es responsable de la inestabilidad genética de estas células, por una parte a través de la regulación que ejerce sobre la formación de focos de reparación de FANCD2 durante la fase S del ciclo celular y por otra, el impacto que generan las aberraciones centrosómicas durante la mitosis.

Aunque existían evidencias previas de que *BCR-ABL1* reprime post-transcripcionalmente los niveles de BRCA1 en LMC, no se conocía con certeza qué

mecanismos operan en la célula para disminuir su expresión. La inhibición de la ruta de la PI3K/AKT y de la actividad del proteosoma restauraron la aparición de focos de BRCA1 y de FANCD2 en los progenitores que expresan *BCR-ABL1* tras el tratamiento con agentes entrecruzantes, sugiriendo una regulación de BRCA1 dependiente de mecanismos de degradación ubiquitina-proteosoma en LMC.

Puesto que los microARNs (miARNs) son reguladores post-transcripcionales clave en las células, estudiamos su posible contribución en la represión de BRCA1 en LMC. En este sentido, los resultados también demostraron que BCR-ABL1 controla los niveles de BRCA1 a través de una ruta alternativa que implica un aumento en la expresión del grupo miR-183-96-182, tanto en progenitores de LMC como en células $CD34^+$ transducidas con un vector que expresa *BCR-ABL1*. La represión de los distintos componentes de este grupo de miARNs mediante ARNs de interferencia expresados con vectores lentivirales (LV:AntimiRs), no sólo incrementó los niveles de BRCA1 en células en proliferación, sino que también aumentó la expresión de dos de los supresores más importantes de la ruta PI3K/AKT, como son FOXO3a y PTEN. Este aumento correlacionó con la disminución de los niveles de radicales superóxido y de la fragilidad cromosómica espontánea en los progenitores *BCR-ABL1*. Además, la inhibición del grupo miR-183-96-182 recuperó la funcionalidad de BRCA1 y FANCD2, que fueron capaces de translocarse a los focos de reparación del ADN en respuesta al daño genotóxico inducido por mitomicina C (MMC), revirtiendo las alteraciones en la ruta de AF/BRCA observadas en las células de LMC. En conjunto, estos resultados indican que el oncogén de fusión *BCR-ABL1*, a través de la desregulación del grupo miR-183-96-182, induce inestabilidad genética en los progenitores *BCR-ABL1*, lo que podría tener consecuencias en relación a la progresión de la enfermedad.

De forma sorprendente, la sobre-expresión del grupo miR-183-96-182 produjo una reducción drástica en la proliferación y en la capacidad clonogénica de los progenitores hematopoyéticos BCR-ABL1. Este efecto se debió, en parte, al incremento en la apoptosis, además de un marcado aumento en la senescencia celular. El estudio de la senescencia reveló que ésta se disparaba de forma prematura por un mecanismo en el que se induce la expresión de p21 y p53, correlacionando con la desaparición de la proteína ubiquitina-ligasa de p53, HDM2. Más aún, aunque demostramos que esta respuesta en los progenitores BCR-ABL1 se realizó en presencia de daño oxidativo en el ADN, no supuso la formación de focos en la heterocromatina asociados a la senescencia (SAHF) ni la activación de la cinasa ATM, por lo que recapituló muchas de las características de la senescencia inducida por pérdida de PTEN (PICS). A nuestro parecer ésta es la primera vez que se caracteriza

una respuesta PICS en progenitores hematopoyéticos. PICS representa una nueva forma de senescencia que no requiere hiper-replicación previa, ni activación de los mecanismos de respuesta a daño en la célula, por lo que podría ser inducida para eliminar células quiescentes BCR-ABL1, encargadas del mantenimiento de la leucemia. Estos resultados sugieren por tanto, que la regulación fina del grupo de miR-183-96-182 no sólo está participando en la acumulación de especies reactivas de oxígeno (ROS) y la respuesta al daño en el ADN, particularmente a través de la ruta de la AF/BRCA, sino que su modulación podría tener también un papel en el control de la senescencia celular, incluso a nivel de las células madre leucémicas (CML), por lo que proponemos este grupo de miRNAs como nuevas dianas para la terapia anti-LMC.

III. INTRODUCCIÓN

1. Características generales de la Leucemia mieloide crónica.

1.1 El cromosoma Filadelfia.

La Leucemia mieloide crónica (LMC) es una enfermedad mieloproliferativa clonal de la célula madre hematopoyética que se caracteriza por la aparición del cromosoma Filadelfia (Ph). Este cromosoma toma su nombre de la ciudad que alberga los dos laboratorios donde fue descubierto. En 1960, Peter Nowell y David Hungeford (Nowell and Hungerford, 1960) describieron la existencia de un cromosoma acrocéntrico anormal en los pacientes con LMC que, al principio, se clasificó como una deleción cromosómica. Más tarde, en 1973, Janet Rowley (Rowley, 1973) describió el cromosoma Ph como un cromosoma 22 reducido, que resultó ser el producto de la translocación recíproca y compensada entre los brazos largos de los cromosomas 9 y 22, t(9:22)(q34;q11) (Figura 1).

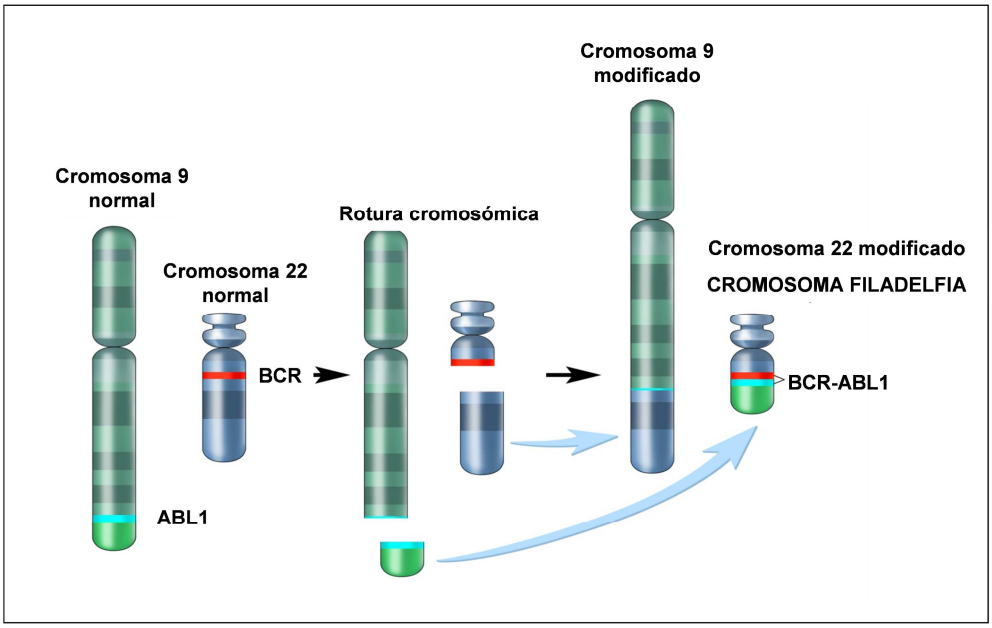

Figura 1. Formación del cromosoma Filadelfia (Ph). (Tomado de Terese Winslow. *The National Cancer Institute's Dictionary of Cancer Terms* . Gobierno de los EE.UU).

Durante los años 70 y 80, la confluencia de las investigaciones con retrovirus transformantes y de los estudios genéticos de estas localizaciones cromosómicas, permitió identificar el gen transformante del virus de la leucemia murina de Abelson, v-*ABL* y la clonación, así como la localización cromosómica de su homólogo celular, c-*ABL1*, en la LMC (Abelson and Rabstein, 1970; Goff et al., 1980; Reddy et al., 1983; Wang et al., 1984). De esta forma, en 1984, se logró describir por primera vez la región BCR (del inglés "*Breakpoint cluster region*") (Groffen et al., 1984). Como

resultado de estos estudios se determinó que la consecuencia molecular de la translocación (9;22) era la yuxtaposición de la secuencia 3' del protooncogén c-*ABL1 (ABL1)* en el cromosoma 9 junto con la secuencia 5´ de *BCR* en el cromosoma 22, que conducía a la aparición del oncogén de fusión *BCR-ABL1* (Figura 2) (Bartram et al., 1983; de Klein et al., 1982).

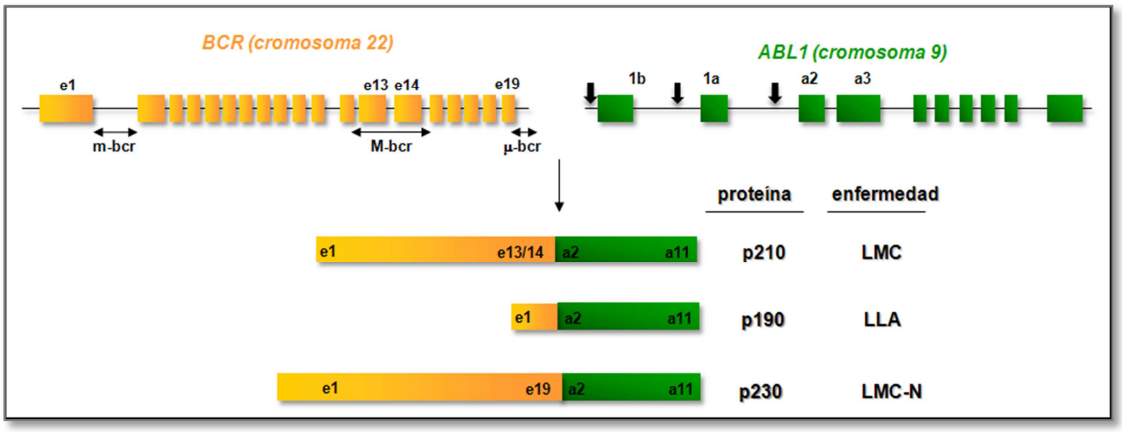

Figura 2. Puntos de ruptura en el oncogén de fusión *BCR-ABL1.* La figura muestra las distintas isoformas del oncogén en la leucemia mieloide crónica, leucemia linfoide aguda y leucemia neutrofílica crónica.

Posteriormente, a finales de los 80, se demostró que el oncogén *BCR-ABL1* codificaba la proteína BCR-ABL1 (Ben-Neriah et al., 1986; Davis et al., 1985; Mes-Masson et al., 1986), una oncoproteína quimérica de mayor tamaño que c-ABL1, traducida a partir de los tránscritos reordenados del gen de fusión, que se caracterizan por primera vez en 1985 (Shtivelman et al., 1985). Esta oncoproteína poseía una actividad tirosina cinasa de expresión constitutiva y potenciada frente a c-ABL1, cuya actividad se encuentra muy regulada en la célula (Konopka and Witte, 1985; Lugo et al., 1990). Estos trabajos también mostraron que la expresión de BCR-ABL1 en progenitores hematopoyéticos resultaba necesaria y suficiente para la transformación celular *in vitro* (McLaughlin et al., 1987).

Pero no fue hasta 1990, en el laboratorio de David Baltimore, donde George Daley y Richard van Etten confirmaron la leucemogenicidad de BCR-ABL1. Expresando el oncogén en células hematopoyéticas en un modelo de ratón, demostraron que la expresión ectópica de *BCR-ABL1*, como un único evento oncogénico, inducía un síndrome mieloproliferativo que recapitulaba muchas de las características de la LMC (Daley et al., 1990). De esta forma, aportaron la prueba definitiva de que la aparición del oncogén de fusión era el agente causante de la enfermedad.

1.2 Leucemias humanas causadas por las diferentes isoformas del oncogén.

Se han identificado tres formas predominantes en el reordenamiento de *BCR-ABL1*. Éstas varían en función de donde se encuentre exactamente el punto de ruptura en *BCR*, y cada una de ellas se asocia de forma preferente a un tipo específico de leucemia (Melo, 1996). Los puntos de ruptura en *c-ABL1* siempre se encuentran en el exón 1, y de forma mayoritaria entre los exones 1a y 1b (Melo, 1996). En el 90% de los pacientes con LMC y en un 30% de los pacientes con leucemia linfoblástica aguda tipo B (LLA-B) Ph+, el punto de ruptura ocurre en *BCR* en una región de 5,8 Kb que comprende los exones 12 a 16 (formalmente b1-b5) y que se denomina M-*bcr* (de *major breakpoint cluster región*). El procesamiento alternativo del ARN en esa región genera los tránscritos de fusión b3a2 (e14a2) o b2a2 (e13a2) que se traducen en una proteína de 210 kDa que incluye 902 ó 909 aminoácidos de BCR, $p210^{BCR\text{-}ABL1}$ (Faderl et al., 1999). En el 60% de los pacientes con LLA-B Ph+ y en casos muy raros de LMC, el punto de ruptura en BCR se localiza en una región de 54,4 Kb entre los exones e2' y e2 que se denomina m-*bcr* (de *minor breakpoint cluster region*), y se generan los tránscritos e1a2 que se traducen en la proteína $p190^{BCR\text{-}ABL1}$ (Melo et al., 1994; Ravandi et al., 1999). Por último, se ha descrito un tercer reordenamiento con el punto de ruptura situado entre los exones e19 y e20. Este área de ruptura, llamada µ-bcr, genera tránscritos de fusión e19a2 que se traducen en la proteína $p230^{BCR\text{-}ABL1}$, presente en la Leucemia neutrofílica crónica (LMC-N) (Figura 2) (Pane et al., 1996). La proteína $p190^{BCR\text{-}ABL1}$, aún siendo la más pequeña, tiene 5 veces más actividad tirosina cinasa que $p210^{BCR\text{-}ABL1}$ (Lugo et al., 1990) y esa potencia correlaciona con la aparición de formas agudas de leucemia. La isoforma $p230^{BCR\text{-}ABL1}$ parece ser el oncogén con menos actividad (Li et al., 1999) y se asocia por ello a la aparición de un síndrome mieloproliferativo benigno, carente de la fase terminal de las leucemias crónicas (Pane et al., 1996).

1.3 Estructura y auto-regulación de la proteína BCR-ABL1.

La proteína $p210^{BCR\text{-}ABL1}$ presenta una estructura modular con diferentes dominios funcionales, suma de los dominios de las proteínas de las que proviene, excepto la secuencia traducida del primer exón de *c-ABL1* y la secuencia de aminoácidos correspondientes a las distintas zonas de ruptura. La proteína $p145^{ABL1}$ es una tirosina cinasa no receptora que contiene en su región terminal un dominio que se denomina *Cap*, presente en las dos isoformas distintas que se generan por procesamiento alternativo del ARN de su primer exón, y que se denominan 1a y 1b. *ABL1b* se expresa mucho más que *ABL1a*, y ambas se diferencian por la presencia en *ABL1b* de

un grupo C14-miristoilo unido covalentemente. Aparte de la región *Cap*, aparecen los dominios de homología conservados de SRC 2 (SH2) y 3(SH3), el dominio tirosina cinasa o SH1, un dominio de unión a proteínas adaptadoras, tres señales de localización nuclear, un dominio de unión a DNA, otro de unión a ACTINA y, finalmente, uno de exportación nuclear, que determinan la localización de la proteína en respuesta a estímulos (Figura 3). $p160^{BCR}$ también presenta una estructura modular. Está compuesto de un dominio de oligomerización *coiled-coil*, seguido de un dominio serina/treonina cinasa, un dominio de homología del factor intercambiador de nucleótidos de guanina Dbl/CDC24, un dominio de homología a PLECSTRINA, un sitio de unión a lípidos calcio-dependiente y finalmente, un dominio de activación para RAC-GTPasas (RAC-GAP) (Deininger et al., 2000). $p160^{BCR}$ contiene un aminoácido Tirosina en la posición 177, clave para el anclaje de proteínas adaptadoras (como GRB2 o la proteina 14-3-3) y esencial para oncogénesis mediada por $p210^{BCR-ABL1}$ (Pendergast et al., 1993)(Figura 3).

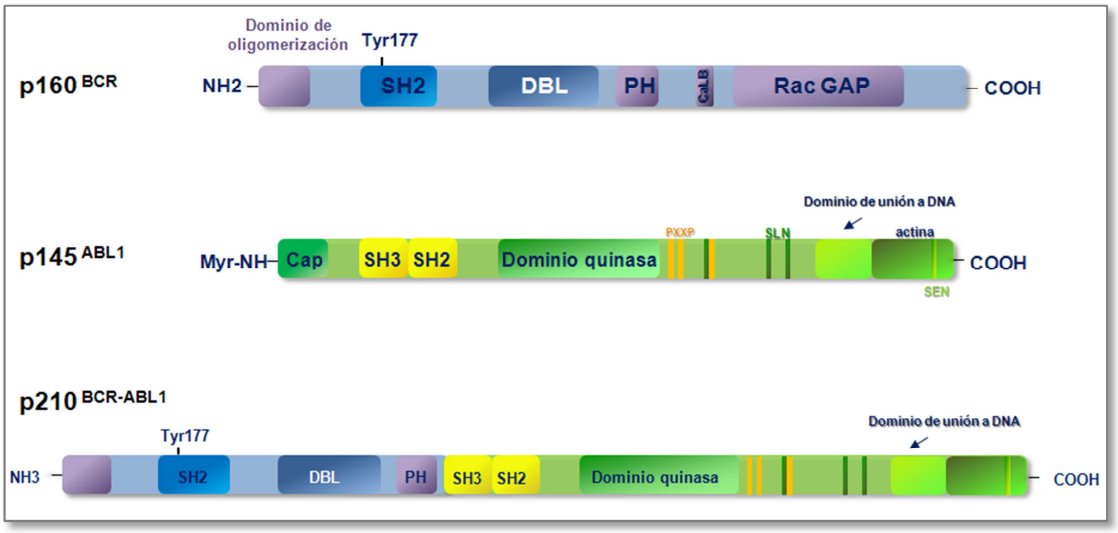

Figura 3. Estructura modular de $p210^{BCR-ABL1}$.

Se han descrito tres eventos moleculares que se relacionan con el aumento exhacerbado de la actividad cinasa y por tanto su capacidad de transformación celular. El primero, está relacionado con la miristolación del extremo N-terminal de ABL1b que induce la autoinhibición de su actividad, porque permite el desplazamiento de los dominios reguladores SH2 y SH3 que conforman una grapa sobre el dominio cinasa. Esto desplaza residuos catalíticos importantes fuera del centro activo y facilita la conformación inactiva de la molécula. El grupo miristoilo está ausente en la fusión $p210^{BCR-ABL1}$, lo que conduce a la desregulación y al incremento de actividad de la oncoproteína de fusión (Hantschel and Superti-Furga, 2004; Nagar et al., 2003). El

segundo evento se debe al dominio de oligomerización de $p210^{BCR-ABL1}$, que permite la generación de dímeros o tetrámeros de la oncoproteína (Zhao et al., 2002). Esta configuración espacial correlaciona directamente con la potenciación de la actividad cinasa que facilita la auto y trans-fosforilación de residuos como la tirosina 177 (Million and Van Etten, 2000) en BCR, también residuos tirosina en el bucle de activación del dominio cinasa y en el dominio de unión entre SH2 y el dominio cinasa de ABL1, modificando la conformación autoinhibida de $p210^{BCR-ABL1}$ (Hantschel and Superti-Furga, 2004; Smith et al., 2003). Las mutaciones en el domino *coiled-coil* impiden la oligomerización y comprometen la actividad cinasa y el potencial leucemogénico mediado por $p210^{BCR-ABL1}$ (McWhirter et al., 1993; Muller et al., 1991; Smith et al., 2003). El tercero y último, se debe al hecho de que secuencias del primer exón de BCR se unen específicamente a la región reguladora SH2 de ABL1 de una forma que no depende de la fosforilación de sus tirosinas, pero que genera una nueva conformación que permite una segunda unión sobre SH2, esencial para la transformación mediada por $p210^{BCR-ABL1}$ (Pendergast et al., 1991). De hecho, se ha determinado que la capacidad del dominio regulador SH2 de ABL1 para estimular la actividad cinasa de la oncoproteína depende de forma crítica del establecimiento de una interfaz estrecha entre el dominio SH2 y el lóbulo N del dominio cinasa (Grebien et al., 2011). La conformación espacial inactiva de $p145^{ABL1}$ y activa de $p210^{BCR-ABL1}$ se muestran en la (Figura 4).

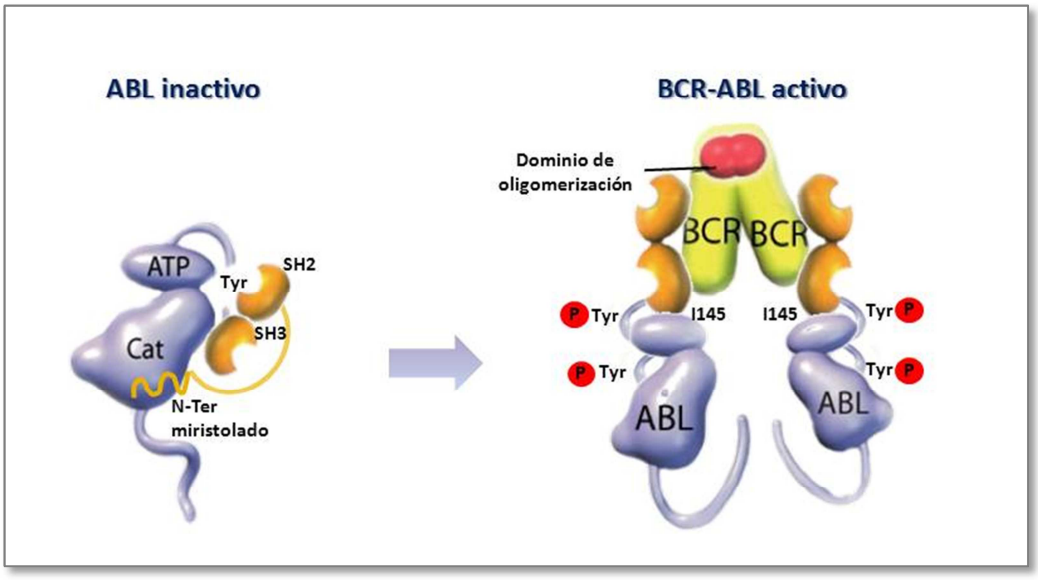

Figura 4. Conformación espacial de la forma inactiva de $p145^{ABL1}$ y de la forma activa de $p210^{BCR-ABL1}$. Modificada de Krause y col. (Krause and Van Etten, 2005).

1.4 Principales rutas de señalización interferidas por BCR-ABL1.

Una vez caracterizada la oncoproteína patogénica $p210^{BCR\text{-}ABL1}$ (en adelante, BCR-ABL1), los esfuerzos se encaminaron hacia la comprensión de los mecanismos moleculares de acción de BCR-ABL1 a través de distintas rutas de señalización intracelular. Debido a su localización citoplásmica (Wetzler et al., 1993), la actividad tirosina cinasa permite a la oncoproteína fosforilar un número muy elevado de sustratos que no son accesibles para c-ABL1 que, aunque puede translocarse entre el núcleo y citoplasma, es de preferente localización nuclear (Van Etten et al., 1989). Se ha demostrado que la actividad tirosina cinasa de c-ABL1 en el núcleo tiene un papel importante en el control de la proliferación celular (Kipreos and Wang, 1990; Sawyers et al., 1992), en la inducción de apoptosis (Agami et al., 1999; Wen et al., 1996) y en la reparación del ADN (Baskaran et al., 1997; Wang, 2000).

Como consecuencia de la expresión ectópica de BCR-ABL1 en líneas celulares dependientes de factores, se ha identificado una plétora de sustratos de BCR-ABL1 en distintas rutas de señalización, que se han relacionado con los cambios fenotípicos que caracterizan a la LMC (Figura 5). De forma resumida, la expresión de BCR-ABL1 conlleva un incremento en la tasa de proliferación celular y la inhibición de la apoptosis en células madre o progenitores hematopoyéticos, que producen un incremento masivo de células mieloides. También se inducen cambios en la adhesión de las células de LMC al estroma de la médula ósea y en su motilidad, que permiten la liberación prematura de células mieloides inmaduras a la circulación y favorecen su invasividad a tejidos. Asimismo, la expresión de BCR-ABL1 puede desregular la respuesta al daño en el ADN, induciendo inestabilidad genética y resistencia a tratamientos genotóxicos en la célula.

Los sustratos fosforilables por BCR-ABL1 incluyen miembros de las rutas de transducción de señales de RAS, fosfatidil-inositol-cinasa 3 (PI3K)/AKT y JAK/STAT.

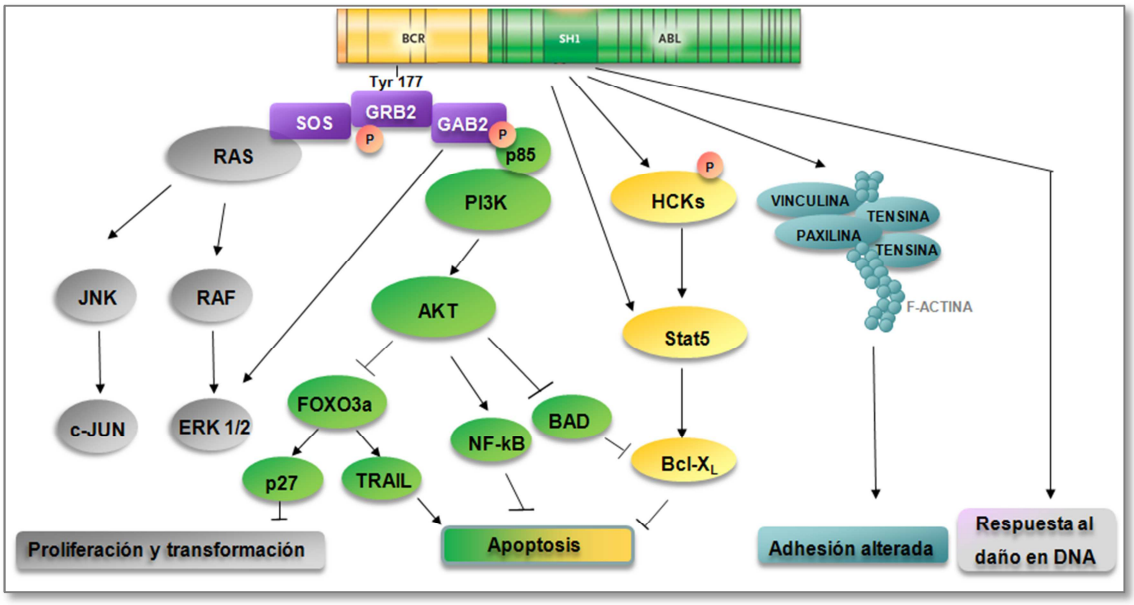

Figura 5. Representación esquemática de las principales rutas de señalización activadas por BCR-ABL1.

La vía de RAS permanece constitutivamente activada por la interacción directa entre la tirosina fosforilada en la posición 177 de BCR-ABL1 y el dominio SH2 de la proteína de unión al factor de crecimiento 2 (GRB2). GRB2 recluta al intercambiador de guaninas SOS y esta unión produce la activación de RAS y del adaptador GAB2. BCR-ABL1 fosforila GAB2 gracias a la formación del complejo GBR2/GAB2. Esta modificación es suficiente para producir la activación constitutiva de la cinasa ERK en células primarias de LMC (Pendergast et al., 1993; Ren, 2005; Sattler et al., 2002). La interferencia de esta ruta mediante oligonucleótidos antisentido, expresión de moléculas dominantes-negativas o inhibición química, suprime la proliferación y la respuesta a estímulos apoptóticos en las células que expresan BCR-ABL1 (Peters et al., 2001; Sawyers et al., 1995; Skorski et al., 1995).

La vía de la PI3K/AKT se activa por BCR-ABL1 de forma indirecta también a través del complejo GBR2/GAB2. La fosforilación de GAB2 induce la activación constitutiva de la PI3K a través de su subunidad p85 (Skorski et al., 1995). En consecuencia, BCR-ABL1 es incapaz de transformar células mieloides primarias de ratones *Gab2*$^{-/-}$ (Sattler et al., 2002). La activación de la PI3K produce una cascada de eventos fundamentales para la transformación celular mediados por la activación de AKT (Franke et al., 1995), regulando la activación o la localización celular de BAD, MDM2, Iκβ-cinasa α y miembros de la familia de factores de transcripción *Forkhead*, como FOXO3a (Mayo and Donner, 2001; Salomoni et al., 2000). En células que expresan BCR-ABL1, se fosforila BAD y queda secuestrada en el citoplasma junto con la proteína 14-3-3β, de

esta forma se suprime su capacidad pro-apoptótica. La fosforilación de MDM2 altera su localización y permite la degradación de p53. La subunidad α de la cinasa Iκβ fosforilada se degrada e induce la activación del factor de transcripción NF-κβ (Ozes et al., 1999; Reuther et al., 1998) y la fosforilación de FOXO3a impide su función en el núcleo (Brunet et al., 1999), donde normalmente transactiva genes que promueven apoptosis (*TRAIL*) (Ghaffari et al., 2003) o inhiben la progresión del ciclo celular (*p27*) (Komatsu et al., 2003). En consecuencia, la inhibición de la ruta de la PI3K/AKT suprime la formación de colonias *in vitro* y la leucemogénesis *in vivo* de las células que expresan BCR-ABL1 (Skorski et al., 1997; Skorski et al., 1995). Sin embargo, aunque BCR-ABL1 a través de la activación de AKT fosforila FOXO3a en las células progenitoras primarias de LMC, promoviendo su retención citoplásmica, su degradación y bloqueando su función pro-apoptótica, resultados recientes confirman que su expresión es nuclear en células madre LMC, superando la señal inhibitoria de AKT mediante la activación de la vía de TGFβ (Naka et al., 2010). FOXO3a en estas condiciones induce el factor de transcripción BCL-6, que facilita la supervivencia de las células reprimiendo P53 y ARF, como parte de la respuesta a estrés inducida para prevenir la apoptosis (Hurtz et al., 2011).

La vía de STAT5 se activa constitutivamente cuando BCR-ABL1 fosforila HCK (Klejman et al., 2002), que es una de las proteínas de la familia de las cinasas SRC (STK), aunque algunos trabajos sugieren que la onco-cinasa puede fosforilar una tirosina clave del dominio SH2 de STAT5 y activarlo directamente (Hantschel et al., 2012). HCK fosforilada recluta y fosforila STAT5 y éste, dimeriza y se transloca al núcleo donde actúa como un factor de transcripción (Ilaria and Van Etten, 1996). STAT5 fosforilado puede aumentar la transcripción génica de la CICLINA D1, que induce la progresión del ciclo celular de la fase G1 a la S (Nosaka et al., 1999). No obstante, uno de sus efectos más relevantes es la activación transcripcional del gen que codifica la proteína anti-apoptótica Bcl-xL (Gesbert and Griffin, 2000). Muchos trabajos han demostrado el papel de la activación de STAT5 en el mantenimiento de la capacidad clonogénica y de transformación *in vitro,* y en la inducción y el mantenimiento de la LMC en ratón (Nieborowska-Skorska et al., 1999; Ye et al., 2006). Sin embargo, el trasplante en ratón de células de médula ósea *Stat5a*$^{-/-}$ y *Stat5b*$^{-/-}$ transducidas con BCR-ABL1, seguía produciendo un síndrome mielodisplásico muy parecido a la LMC(Sexl et al., 2000). Posteriormente, se determinó que las mutaciones en *Stat5* manejadas en este trabajo eran hipomórficas, con lo que no suprimían la expresión del factor de transcripción de forma completa (Bunting et al., 2002). En la actualidad, existen evidencias suficientes que indican que la señalización vía STAT5

es imprescindible para la leucemogénesis y el mantenimiento de la LMC (Hoelbl et al., 2010; Ye et al., 2006).

Por último, y de manera significativa, la localización citosólica de BCR-ABL1 permite aumentar su capacidad de unión a ACTINA, comparado con c-ABL1,(McWhirter and Wang, 1993; Salgia et al., 1997) y fosforilar algunas proteínas citoesqueléticas, como FAK (Gotoh et al., 1995) o PAXILINA (Salgia et al., 1995). BCR-ABL1 induce el procesamiento alternativo del ARNm de la tirosina cinasa 2 rica en prolina (PYK2), aumentando la expresión de la INTEGRINA β1, que contribuye a la adhesión aberrante de los progenitores LMC (Salesse et al., 2004). En conjunto, estos efectos contribuyen a una alteración en la adhesión de las células, que puede explicar la liberación prematura de progenitores y precursores a la circulación en pacientes con LMC (Bhatia and Verfaillie, 1998; Gordon et al., 1987; Pelletier et al., 2004).

1.5 Epidemiología y aspectos clínicos.

La LMC es la patología neoplásica más estudiada y es un modelo de progresión tumoral para otros cánceres. Además, es la primera neoplasia humana que se asoció de forma consistente a una única anomalía cromosómica adquirida. Es también, la primera enfermedad donde se ensayó la terapia molecular dirigida. Comprende el 15% del total de las leucemias adultas y presenta una incidencia aproximada de 1 a 1,5 casos cada 100.000 habitantes/año. La media de edad al diagnóstico se sitúa entre los 50 y 60 años de edad en los países de Occidente, y los hombres se ven más afectados por la enfermedad que las mujeres con una relación de 1,4:1. No se han detectado diferencias geográficas o étnicas en la aparición de la enfermedad (An et al., 2010; Melo and Barnes, 2007; Perrotti et al., 2010). No se conocen en la actualidad las causas por la que aparece la translocación, aunque algunos autores lo asociaron al aumento de radiación ionizante (Deininger et al., 1998; Preston et al., 1994). Sin embargo, la mayoría de casos de LMC no tienen un historial de alta exposición a radiación o leucemógenos químicos. Se ha propuesto como explicación la proximidad espacial de los genes no homólogos BCR y ABL1 en el núcleo interfásico de las células de médula ósea (Lukasova et al., 1997).

La LMC es una enfermedad que se manifiesta en tres fases distintas de forma secuencial (Figura 6):

-Fase Crónica (FC-LMC): es la fase indolente, de larga duración (5-6 años), que se caracteriza por la aparición de leucocitosis, con acumulación masiva de granulocitos maduros y un incremento en el número de progenitores mieloides en médula ósea,

sangre periférica y regiones extramedulares. Más del 90% de los pacientes con LMC son diagnosticados en esta fase. La enfermedad debuta con algunos síntomas que incluyen astenia, pérdida de peso, hemorragias, sudoración, anemia y esplenomegalia, pero hasta el 50% de los pacientes pueden ser asintomáticos y se diagnostican en análisis de sangre realizados por causas no relacionadas.

-Fase acelerada (FA-LMC): No aparece aproximadamente en un 20% de los pacientes, que transitan directamente a la fase blástica. De una duración aproximada de 6 meses. Se caracteriza por un aumento en la proporción de células progenitoras frente a células hematopoyéticas maduras. Los criterios que definen esta fase son controvertidos y siguen siendo materia de debate. Pueden incluir la presencia de un 10–19% de mieloblastos o más del 20% de basófilos en sangre periférica o médula ósea, trombocitopenia persistente no relacionada con el tratamiento, evolución citogenética y mayor esplenomegalia.

-Fase blástica (CB-LMC): Fase agresiva hacia la que todos los pacientes sin tratamiento progresan y que, en esas condiciones, resulta fatal. La supervivencia media de los pacientes en esta fase es de 3-6 meses. Se caracteriza por la expansión rápida de poblaciones de blastos mieloides o linfoides con la diferenciación bloqueada, que se acumulan (>20%) en la médula y en sangre periférica e infiltran regiones extramedulares como la piel, huesos o nódulos linfáticos (Baccarani et al., 2013; Jabbour and Kantarjian, 2014; Melo and Barnes, 2007).

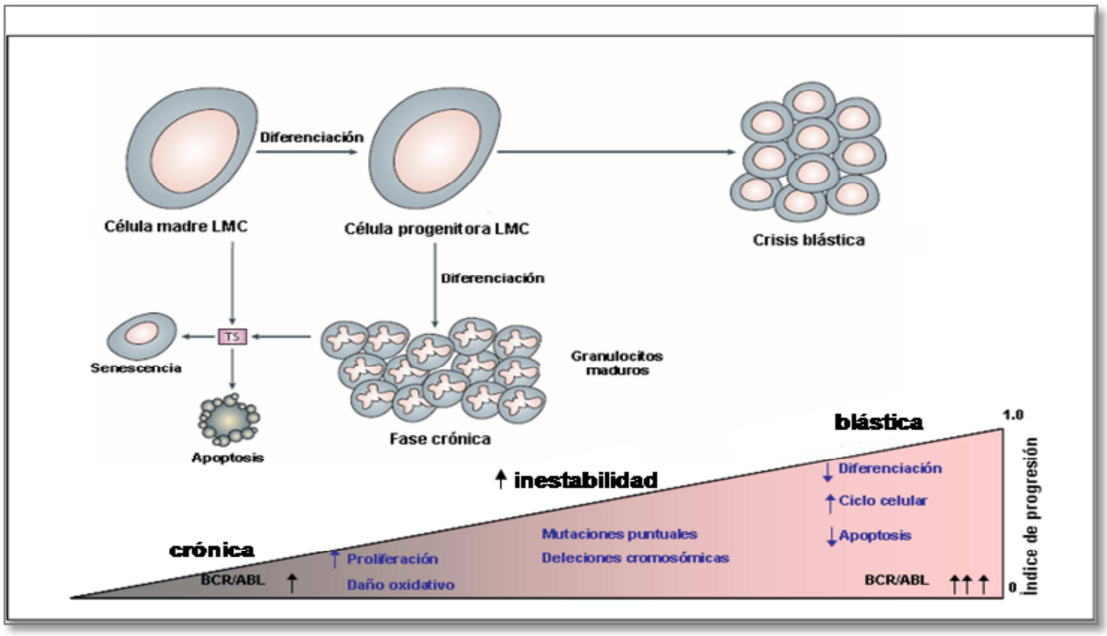

Figura 6: Progresión de la enfermedad en la LMC. Tomado y modificado de Melo y col. (Melo and Barnes, 2007).

1.6 Primera línea de tratamiento en LMC. Inhibidores de tirosina cinasas: Imatinib.

Las opciones terapéuticas para la LMC antes del descubrimiento del oncogén BCR-ABL1 incluían agentes citotóxicos como el busulfan y la hidroxiurea (Bolin et al., 1982). Aunque esta quimioterapia lograba normalizar los contajes hematológicos y controlar algunas de la manifestaciones clínicas de la LMC, no conseguía variar la progresión natural de la enfermedad hacia la CB-LMC (Faderl et al., 1999). Con la introducción del interferón-alfa recombinante (rIFN-α) en la terapia se logró un aumento significativo en la supervivencia de los pacientes, sin embargo, sólo una minoría de éstos mostraba una remisión citogénetica completa y muchos no toleraban sus efectos adversos (Silver et al., 1999). El trasplante hematopoyético alogénico es la única aproximación curativa de la enfermedad. No obstante, la morbilidad y mortalidad asociada al trasplante de pacientes con edades en promedio superiores a los 50 años, combinadas con la dificultad para disponer de un donante compatible, limitaba esta opción a una proporción reducida de pacientes (<30%) (Baccarani et al., 2006). En este contexto, una vez desentrañado que las funciones transformantes de BCR-ABL1 dependían de su actividad tirosina cinasa, Nick Lydon, Brian Druker y Charles Sawyers desarrollan el fármaco imatinib (STI571) a finales de los años 90 (Druker et al., 1996). El compuesto se obtuvo mediante rastreo masivo de moléculas químicas que podían inhibir la proteína oncogénica y supone el primer ejemplo de diseño racional de fármacos. Es una 2-fenilaminopirimidina modificada que actúa como inhibidor competitivo del ATP. Se une a la configuración inactiva de la tirosina cinasa, bloquea la región de unión a ATP e impide el cambio conformacional a su forma activa (Savage and Antman, 2002)(Figura 7). Como consecuencia, inhibe la fosforilación de sus sustratos e impide el crecimiento del clon leucémico. La molécula también inhibe el receptor del factor de crecimiento derivado de plaquetas (PDGFR), ARG y c-KIT (Druker et al., 1996).

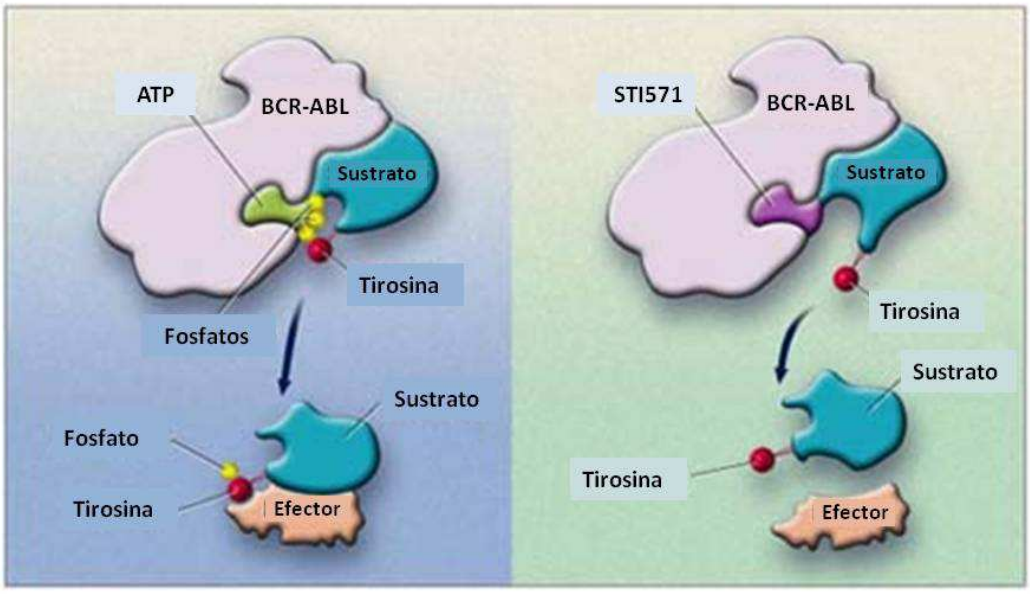

Figura 7: Mecanismo de acción de imatinib. Tomado y modificado de Goldman y col. (Goldman and Melo, 2001).

El mesilato de imatinib (Glevec® Novartis) como agente único mostró una muy alta eficacia terapéutica en pacientes en FC-LMC al diagnóstico, con un 97% de remisiones hematológicas y un 79% de respuestas citogenéticas completas tras 19 meses de tratamiento (O'Brien et al., 2003), por lo que fue aprobado en el año 2001 por la FDA como tratamiento en primera línea para pacientes en esta fase (Cohen et al., 2002). Desde entonces y hasta la fecha, imatinib ha dominado la estrategia terapéutica en LMC como estándar sin discusión y ha conseguido cambiar la historia natural de la enfermedad, variando la supervivencia general de los pacientes a 10 años, de un 20% a un 80-90% (Jabbour and Kantarjian, 2014). Imatinib también ha favorecido el desarrollo de otras moléculas pequeñas inhibidoras de tirosina cinasas (TKI), tanto de segunda como de tercera generación. Esto último, se debe a que imatinib no es una droga perfecta. Aunque el seguimiento a 8 años reveló unas tasas de respuesta citogénetica completa que variaban entre un 66-88% y una supervivencia general de entre 83-97%, aproximadamente un 45% de los pacientes no continuaba el tratamiento por falta de eficacia o por la aparición de eventos adversos (Baccarani et al., 2013; Jabbour and Kantarjian, 2014), por lo que se ensayaron frente a imatinib, dos TKI más potentes, dasatinib (Sprycel® Bristol-Myers Squibb) y nilotinib (Tasigna® Novartis). Nilotinib es una molécula derivada de imatinib, con el mismo mecanismo de acción pero 30 veces más potente inhibiendo la actividad cinasa de BCR-ABL1 *in vitro*. Dasatinib es capaz de inhibir la actividad de la cinasa BCR-ABL1 y de las cinasas de la familia SRC, junto con otras cinasas oncogénicas incluyendo c-KIT, los receptores

cinasa de las efrinas (EPH) y el receptor del PDGF. Dasatinib es un inhibidor de la cinasa 300 veces más potente que imatinib, que es capaz de unirse tanto a la conformación activa como inactiva de BCR-ABL1 (O'Hare et al., 2005; O'Hare et al., 2012). Los ensayos clínicos que compararon dasatinib vs imatinib (DASISION) (Kantarjian et al., 2010) y nilotinib vs imatinib (ENESTnd) (Saglio et al., 2010), demostraron que los grupos experimentales fueron superiores con respecto a la respuesta citogenética y molecular completa, no así frente a parámetros de supervivencia, por lo que en el año 2010 ambos TKI también fueron aprobados como primera línea de tratamiento en FC-LMC junto con imatinib.

La principal razón por la que se produce el fracaso del tratamiento con TKI o la aparición de recaídas en la enfermedad es la aparición de resistencias, con el consecuente riesgo de progresión a fases más avanzadas con muy mal pronóstico. Estas resistencias pueden depender de la actividad de BCR-ABL1 o no depender en absoluto de su actividad cinasa. Aproximadamente el 50% de los pacientes resistentes a los TKI aprobados presentan una mutación puntual en alguno de los más de 80 residuos estudiados en el dominio tirosina cinasa de BCR-ABL1 que interfieren con la unión del fármaco (Baccarani et al., 2013; O'Hare et al., 2012). Mucho antes de ser utilizados en primera línea, dasatinib y nilotinib fueron aprobados como segunda línea de tratamiento en LMC para pacientes intolerantes o resistentes a imatinib basados en unas tasas de respuesta citogenéticas del 40% y 60% y unas respuestas moleculares del 42% y del 28%, tras dos y cinco años, respectivamente (Giles et al., 2013; Kantarjian et al., 2011; Shah et al., 2010). Estos inhibidores de segunda generación controlan la mayoría de las mutaciones que confieren resistencia a imatinib, con muy pocas pero notables excepciones, como la mutación T315I o "*gatekeeper*" (O'Hare et al., 2012). El TKI de tercera generación ponatinib (Inclusig® Ariad) (Cortes et al., 2012) es el único inhibidor aprobado como opción terapéutica que controla esta resistencia, pero fue retirado del mercado en octubre del 2013 (Dalzell, 2013) y, posteriormente reducida su indicación a consecuencia de la detección de toxicidad vascular grave tras su comercialización. A pesar de todo este potencial farmacológico, un 20% de pacientes en FC-LMC no responden ni a imatinib ni a los subsiguientes TKI de segunda generación (O'Hare et al., 2012).

A diferencia de la anterior, la resistencia independiente de BCR-ABL1 ocurre en la célula a pesar de la inhibición de su actividad cinasa. Las células madre leucémicas (CMLs) en LMC basan su proliferación y supervivencia en rutas de señalización similares a las responsables de la resistencia a TKI independiente de BCR-ABL1

(O'Hare et al., 2012). El descubrimiento en el año 2011 de que los TKI son incapaces de erradicar las CMLs que mantienen la enfermedad (Bhatia et al., 2003; Graham et al., 2002) a pesar de inhibir por completo la actividad cinasa de BCR-ABL1 (Corbin et al., 2011; Hamilton et al., 2011), junto con el hecho de que la discontinuación de las terapias con TKI inducen recaídas en el 61% de los pacientes en los primeros 7 meses de interrupción del tratamiento (Mahon et al., 2010), con lo que no resultan definitivamente curativas, ha multiplicado los trabajos preclínicos que tratan de conocer qué rutas controlan específicamente la supervivencia de esas poblaciones celulares para eliminarlas, erradicando definitivamente la leucemia o mejorando la efectividad de las terapias con TKI (O'Hare et al., 2012). Junto a los TKI de segunda y tercera generación, durante la última década se están llevando a cabo en paralelo múltiples estrategias para superar la resistencia a TKI en primera línea destacando: inhibidores como agentes únicos o en combinación junto con TKI de moléculas clave en las rutas de señalización independientes de la señal de BCR-ABL1 (Bellodi et al., 2009; Chen et al., 2013; Chen et al., 2009a; Neviani et al., 2013), desestabilizadores de la oncoproteína (Radujkovic et al., 2005), vacunas en CML (Biernacki et al., 2010; Bocchia et al., 2005; Pinilla-Ibarz et al., 2000), inhibidores de la oligomerización de BCR-ABL1 (Dixon et al., 2011) o moléculas que inducen el atrapamiento de la oncoproteína en el núcleo (Vigneri and Wang, 2001) o en agregosomas (Sun et al., 2011).

2. Características de la fase blástica en la LMC.

De forma general, se acepta que la adquisición del oncogén BCR-ABL1 es el evento inicial en la génesis de la FC-LMC, a pesar de algunas evidencias circunstanciales pero recurrentes, que sugieren que la hematopoyesis podría ser clonal en los pacientes antes de la aparición del cromosoma Ph (Fialkow et al., 1981; O'Dwyer et al., 2003; Raskind et al., 1993; Zaccaria et al., 2007). Durante la FC-LMC, la aparición de la fusión ocurriría en una célula madre hematopoyética, que obtendría ventaja proliferativa y/o una capacidad de diferenciación aberrante comparado con células normales, provocando la expansión masiva del compartimento mieloide (Bruns et al., 2009). Sin embargo, los clones blásticos pueden originarse a nivel de un progenitor leucémico más comprometido que adquiriría capacidad de auto-renovación (Jamieson et al., 2004a). La expresión/actividad sostenida de BCR-ABL1 sería la responsable de la inducción de los cambios moleculares y cromosómicos secundarios necesarios para la aparición del fenotipo agresivo que caracteriza a los clones blásticos (Melo and Barnes, 2007; Perrotti et al., 2010).

Las alteraciones genéticas y epigenéticas secundarias a la formación del oncogén BCR-ABL1, que se detectan durante la CB-LMC, son muy comunes. Sin embargo, no existe una única mutación específica en la mayoría de los pacientes a la que se le pueda atribuir la progresión de la enfermedad. Es más probable que la CB-LMC surja de la acumulación de un número crítico de combinaciones de diferentes mutaciones (Perrotti et al., 2010). Las lesiones genéticas que se observan en los pacientes en CB-LMC incluyen la presencia de cromosomas adicionales, deleciones, inserciones y/o mutaciones puntuales (Fabarius et al., 2007; Roche-Lestienne et al., 2008; Yamamoto et al., 2005b), aunque estos patrones difieren en la transformación mieloblástica o linfoblástica (Bacher et al., 2005).

2.1 Principales alteraciones moleculares y cromosómicas mediadas por BCR-ABL1.

A nivel molecular, la mutaciones más comunes (sin tener en cuenta las mutaciones del dominio cinasa de BCR-ABL1), ocurren en los loci de *p53* (20-30% de los casos) y *RUNX1* (38% casos), en la CB-LMC mieloide, y en los loci de *CDKN2A/B* (50% de los casos) y el factor de transcripción Ikaros (*IKZF1*) (55% de los casos), en la CB-CML linfoide (Lanza and Bi, 1995; Mullighan et al., 2008a; Mullighan et al., 2008b; Nakayama et al., 1999; Roche-Lestienne et al., 2008; Sill et al., 1995).

Los cambios epigenéticos dependen directamente de los efectos pleiotrópicos de la actividad constitutiva cinasa (Calabretta and Perrotti, 2004; Melo and Barnes, 2007), que empieza a incrementarse en la FA-LMC (Gaiger et al., 1995). BCR-ABL1 perturba profundamente el transcriptoma celular (Yong and Melo, 2009), alterando la expresión de genes que tienen una participación importante en esta fase, por ejemplo, *PRAME*, *MZF1*, *EVI-1*, *WT1* y *JUNB* (Calabretta and Perrotti, 2004; Oehler et al., 2009; Radich et al., 2006). La expresión del oncogén de fusión también es responsable de los efectos post-transcripcionales, traduccionales y post-traduccionales que inducen la activación constitutiva de factores mitogénicos, antiapoptóticos y de antidiferenciación, como por ejemplo $MAPK^{ERK1/2}$, MYC, JAK2, YES-1, LYN, hnRNP-E2, MDM2, STAT5, BMI-1 y BCL2) o la inhibición de los reguladores más importantes de los procesos celulares como p53, C/EBPα y PP2A (Calabretta and Perrotti, 2004; Melo and Barnes, 2007).

El 80% de los pacientes con LMC presentan aberraciones citogenéticas no aleatorias adicionales a la presencia del cromosoma Ph. A este fenotipo se le denomina “evolución clonal” y es el reflejo de la inestabilidad genética que caracteriza la transición a la CB-LMC (Cortes and O'Dwyer, 2004). Las aberraciones citogenéticas

más frecuentes en los pacientes con evolución clonal son la trisomía del cromosoma 8 (34%), el isocromosoma 17 (20%) y la duplicación del cromosoma Ph (38%) (Johansson et al., 2002), y éstas se han asociado a la sobrexpresión del oncogén *MYC*, pérdida de una copia del gen supresor de tumores *p53* y la sobrexpresión de *BCR-ABL1*, respectivamente (Calabretta and Perrotti, 2004; Gaiger et al., 1995). Entre el 10 y el 15% de los pacientes con LMC presentan deleciones en el cromosoma 9. Esta deleción tiene valor pronóstico, ya que se ha asociado con una progresión más rápida hacia la fase blástica (Huntly et al., 2003). También se han descrito otras aberraciones, como la trisomía 19, la trisomía 21, la trisomía 17 y deleciones en el cromosoma 7 en menos del 10% de los pacientes con evolución clonal (Quintas-Cardama and Cortes, 2006).

2.2 Mecanismos responsables de la progresión de la enfermedad.

Los mecanismos genéticos y epigéneticos responsables de la progresión y transformación a la CB-LMC son muy complejos y apenas se empiezan a conocer. El incremento en la expresión de *BCR-ABL1* va a inducir los mecanismos que participan en el bloqueo en la diferenciación mieloide, el aumento de la proliferación y supervivencia, la adquisición de la capacidad de autorenovación, la inhibición de los genes supresores de tumores y la generación de inestabilidad genética en la célula de LMC (Figura 8).

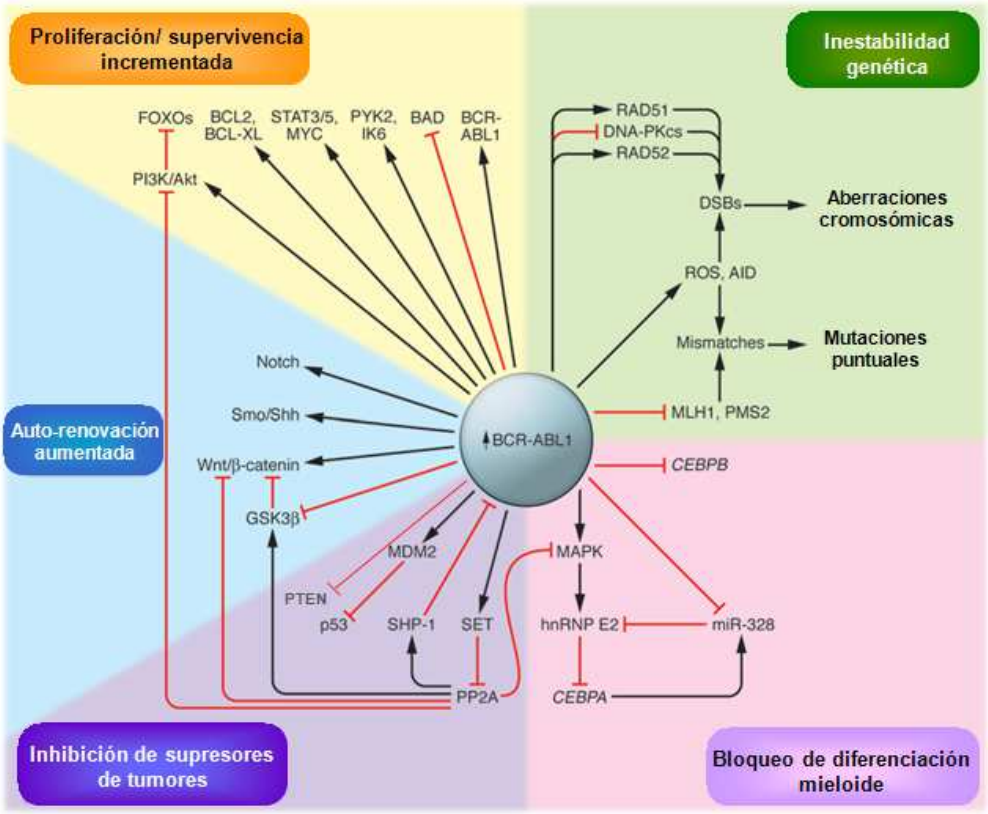

Figura 8: Mecanismos implicados en la transformación blástica dependientes de BCR-ABL1. Tomado y modificado de Perroti y col. (Perrotti et al., 2010).

2.2.1) Incremento en la expresión de *BCR-ABL1.*

El aumento de expresión/actividad de BCR-ABL1 es fundamental no sólo para el mantenimiento del fenotipo neoplásico de la LMC sino también para su progresión. De hecho, las cantidades de ARN mensajero y de proteína de fusión varían con la progresión de la enfermedad y son mucho mayores en células en CB-LMC que en FC-LMC (Gaiger et al., 1995; Guo et al., 1991). En este sentido, se ha detectado que el nivel transcripcional de *BCR-ABL1* es doscientas veces más alto en los progenitores $CD34^+$ que en células diferenciadas (Barnes et al., 2005). Estudios con líneas celulares de FC-LMC y CB-LMC muestran que, *in vitro*, los niveles de BCR-ABL1 modulan la dependencia de las células de LMC a factores de crecimiento (Barnes et al., 2005), la protección contra la apoptosis (Cambier et al., 1998), la motilidad de la célula (Barnes et al., 2005) y también su clonogenicidad (Barnes et al., 2005; Cambier et al., 1998). No se conocen exactamente los mecanismos por los que se produce este incremento de expresión de la fusión oncogénica pero se asume que podría existir una presión selectiva que favorece la expansión de los clones leucémicos más proliferativos y menos diferenciados que expresan mayores niveles de la oncoproteína. A favor de esta hipótesis, se han encontrado niveles mayores de tránscritos de *BCR-*

ABL1 en la subpoblación de progenitores granulo-macrofágicos (PGM) $CD34^+$ de pacientes en CB-LMC, frente a las mismas células de pacientes en FC-LMC (Jamieson et al., 2004a). Esta subpoblación está muy expandida en la CB-LMC y se ha propuesto como candidata a sostener la leucemia durante esta fase (Jamieson et al., 2004a). De forma alternativa, se ha hipotetizado que es la misma proteína BCR-ABL1 la que induce la expresión de los tránscritos de *BCR-ABL1* y/o que la degradación de esos tránscritos está modulada a la baja en progenitores LMC $CD34^+$ (Jiang et al., 2007).

2.2.2) Mecanismos implicados en el bloqueo en la diferenciación mieloide.

Una de las características más importantes de la progresión a la fase blástica es el bloqueo gradual en la diferenciación. El efecto inhibitorio de la alta expresión de BCR-ABL1 sobre la diferenciación se debe en gran parte a la desaparición de C/EBPα, que es el factor de transcripción esencial para la diferenciación granulocítica (Radomska et al., 1998). C/EBPα se expresa en células de médula ósea normales y células en FC-LMC, no así en células de CB-LMC (Perrotti et al., 2002). La expresión ectópica de C/EBPα induce la maduración en precursores mieloides Ph^+ y progenitores $CD34^+$ en CB-LMC (Ferrari-Amorotti et al., 2006; Perrotti et al., 2002; Schuster et al., 2003). Asimismo, se puede inducir una CB-LMC en ratón cuando éstos son transplantados con células de hígado fetal C/EBP$\alpha^{-/-}$ transducidas con *BCR-ABL1* (Wagner et al., 2006). La pérdida de C/EBPα en los PGM FB-LMC no se debe a mutaciones en el gen correspondiente *CEBPA* (Pabst et al., 2006), sino que está mediada a nivel traduccional por los niveles altos de *BCR-ABL1,* a través de la estabilización de ribonucleoproteína hnRNP-E2 (Perrotti et al., 2002).

La expresión de hnRNP-E2 es baja o indetectable en la FC-LMC y muy alta en progenitores CB-LMC, donde suprime la expresión de C/EBPα e inhibe la diferenciación (Perrotti et al., 2002). La capacidad de hnRNP-E2 para inhibir C/EBPα requiere la activación constitutiva de las MAPKs ERK1 y ERK2 (Chang et al., 2007; Notari et al., 2006), que aumentan su estabilidad directamente (Chang et al., 2007). Se puede detectar la activación constitutiva de MAPK en progenitores CB-LMC (Notari et al., 2006). Sin embargo, en progenitores FC-LMC la activación de estas MAPKs es transitoria en respuesta a señales mitogénicas o de supervivencia inducidas por factores de crecimiento extracelulares (Chu et al., 2004). En este sentido, se conoce que altos niveles de expresión de BCR-ABL1 correlacionan con un incremento en la actividad ERK1/2 (Notari et al., 2006). Uno de los reguladores más importantes del eje BCR-ABL1/MAPK/hnRNP-E2 es el micro-ARN-328 (Eiring et al., 2010). Este miARN

se une a los tránscritos de hnRNP-E2 y por tanto impide su interación con el ARN del gen *CEBPA*, restaurando la expresión de C/EBPα en la célula. La pérdida del miR-328 es frecuente en progenitores en la CB-LMC, no así en progenitores mieloides en FC-LMC (Eiring et al., 2010).

De forma ocasional (<5% de los casos), la cooperación de *BCR-ABL1* con otras translocaciones secundarias como *AML1-EVI1* (Nucifora et al., 1993) o *NUP98/HOXA9* (Dash et al., 2002), tienen un papel importante en el desarrollo del fenotipo blástico. AML1-EVI1 como segundo evento oncogénico es capaz directamente de bloquear la diferenciación en la célula (Nucifora et al., 1993). NUP98/HOXA9 desregula el balance normal entre la división simétrica y asimétrica en la célula, favoreciendo la simétrica y por tanto, el crecimiento preferente de precursores inmaduros que caracterizan a la CB-LMC (Wu et al., 2007).

2.2.3) Aumento de la proliferación y supervivencia celular.

Gran parte de las rutas de señalización de BCR-ABL1 que afectan al aumento de la proliferación/supervivencia para el mantenimiento de la célula LMC, ya han sido analizadas en un apartado previo (Ver Principales rutas de señalización interferidas por BCR-ABL1). Estas mismas rutas (RAS, PI3K/AKT y STAT5) son sobre-estimuladas por los altos niveles de BCR-ABL1 que caracterizan a la CB-LMC y, en consecuencia, la fosforilación de sus sustratos también contribuye a la progresión de la enfermedad.

La resistencia a la apoptosis, que es una propiedad intrínseca a la célula madre hematopoyética (CMH), también lo es para la célula madre leucémica (CML). Estudios recientes han demostrado que la inducción de la apoptosis correlaciona con la magnitud y duración de la inhibición de la actividad cinasa de BCR-ABL1 (Shah et al., 2008). Uno de los genes más estudiados en relación a la resistencia incrementada a la apoptosis es *BCL2*. La sobreexpresión de *BCR-ABL1* junto con *BCL2* en un modelo transgénico de ratón induce el desarrollo de una CB-LMC (Jaiswal et al., 2003). El análisis extensivo de marcadores SNP en pacientes demuestra que el SNP (rs1801018) en el gen *BCL2* o los cambios -27A>G y -11G>A en el promotor del gen de la muerte celular programada 5 (*PDCD5*) se asocian a la susceptibilidad a padecer LMC (Kim et al., 2009a; Ma et al., 2005). BCR-ABL1 aumenta la expresión del factor anti-apoptótico Bcl-xL, a través de su inducción por STAT5 (Horita et al., 2000). Bcl-xL no se requiere para el desarrollo y el mantenimiento de la LMC, pero si es esencial para la progresión hacia la CB-LMC, como se ha demostrado recientemente en un

modelo de ratón (Harb et al., 2013). La expresión de *BIM*, un miembro pro-apoptótico de la familia de *BCL2*, es muy baja en líneas celulares en CB-LMC y puede ser inducida mediante la inhibición de la actividad cinasa de BCR-ABL1 (Kuribara et al., 2004).

JAK-2 es una tirosín-cinasa que transduce señales provenientes de receptores de citocinas en la vía JAK/STAT y se ha encontrado fosforilada en células Ph^+ independientes de citocinas (Xie et al., 2002). Un mecanismo descubierto recientemente para facilitar la supervivencia de las CMLs en CB-LMC es la activación de la cinasa LYN vía JAK-2. Este es un mecanismo en el que está directamente implicada la ruta de SET/PP2A/SHP1. La inhibición farmacológica de JAK-2 como agente único o en combinación con TKI induce mucha más apoptosis en progenitores CB-LMC que en progenitores normales (Samanta et al., 2009). Sin embargo, Hantschel et al. han demostrado que la transformación mieloide y el mantenimiento de la leucemia es independiente de JAK-2 en un modelo de eliminación condicional de la tirosín-cinasa (Hantschel et al., 2012). Es más, los inhibidores de JAK-2 eran capaces de inducir apoptosis en las células independientemente de la expresión de JAK-2, por lo que se está cuestionando su especificidad (Hantschel et al., 2012). Una aproximación terapéutica distinta ha consistido en la inhibición de la lipoxigenasa ALOX5, que impide la supervivencia de las CMLs en un modelo de ratón de LMC (Chen et al., 2009a). BCR-ABL1 aumenta los niveles y actividad de ALOX5 independientemente de la actividad cinasa. ALOX5 impide a su vez la expresión de un gen que reprime la proliferación y promueve la apoptosis en las CMLs de LMC, *MSR1* (Chen et al., 2011). El gen de la leucemia promielocítica (PML) es un conocido supresor tumoral pero resulta esencial para el mantenimiento de la CML en LMC (Ito et al., 2008). Finalmente, el descubrimiento de que los progenitores CB-LMC inducen autofagia como mecanismo de resistencia a la apoptosis, ha identificado a los genes que participan en este proceso celular como dianas terapéuticas, ya que su supresión causa la muerte de estas células. En la misma línea, inhibidores del proceso autofágico, como la cloroquina o hidroxicloroquina, pueden potenciar el efecto terapéutico de los inhibidores de la cinasa BCR-ABL1 (Bellodi et al., 2009). De hecho, un estudio reciente en cuatro pacientes con CB-LMC ha demostrado que es efectiva la combinación del inhibidor autofágico claritromicina, junto con el TKI dasatinib (Carella et al., 2012).

2.2.4) Adquisición de la capacidad de autorenovación.

A diferencia de otros oncogenes de fusión implicados en el desarrollo de leucemias humanas (por ejemplo, *MLL-ENL* ó *MOZ-TIF2*), BCR-ABL1 puede transformar CMHs, pero no resulta suficiente para transformar progenitores comprometidos que carezcan de capacidad de autorenovación inherente (Cozzio et al., 2003; Huntly et al., 2004). Es decir, BCR-ABL1 no puede conferir propiedades de autorenovación *per se*. Durante la transición de la FC-LMC a la CB-LMC, las CMLs adquieren anomalías genéticas o epigenéticas adicionales que permiten extender su potencial replicativo (Jamieson et al., 2004b). Se ha observado que en los ratones deficientes en *Junb/Ap-1* (Passegue et al., 2004) o *Iscbp* (Holtschke et al., 1996) se desarrolla un síndrome mieloproliferativo que mimetiza una LMC, con un aumento de la proliferación de CMHs y una expansión mieloide que emula una CB-LMC. No se conoce actualmente si BCR-ABL1 interfiere la expresión de estos factores de transcripción, o si son eventos estocásticos que se producen durante la progresión de la enfermedad.

En otro modelo de ratón en el que la expresión de BCR-ABL1 se restringe a los PGM y su progenie mieloide, pero no a la CMH, se origina un fenotipo de LMC que contiene FA-LMC y CB-LMC. Estas observaciones podrían indicar que la crisis blástica proviene de la adquisición progresiva de alteraciones genéticas en un progenitor comprometido que adquiere propiedades de auto-renovación, como ya se indicó anteriormente (Jaiswal et al., 2003). En humanos, la progresión a la CB-LMC se asocia con una expansión de entre 6 y 10 veces de los PGM, no de las CMLs quiescentes, que constituyen aproximadamente el 0,5% de la población $CD34^+$ total. Por tanto, en los pacientes en CB-LMC podrían coexistir dos poblaciones distintas con capacidad de autorenovación. Una población compuesta de PGM responsable de la expansión rápida de blastos y otra población de CMHs BCR-ABL1 quiescente, encargada de mantener el fenotipo de la FC-LMC (Perrotti et al., 2010).

La autorenovación de los PGM requiere la activación de la ruta de WNT/ß-CATENINA, Sonic Hedgehog y la señalización de Notch. La CB-LMC se asocia con la acumulación de ß-CATENINA en el núcleo de los precursores, otorgándoles potencial de autorenovación (Jamieson et al., 2004a; Minami et al., 2008). En ratón, BCR-ABL1 estabiliza ß-CATENINA a través de la fosforilación de sus tirosinas 86 y 654. La fosforilación inhibe su unión con el tándem AXINA/GSK3ß y permite la interacción con el factor de transcripción 4 de la célula T (TCF4) y LEF1. Estos últimos activan la transcripción de genes que inducen proliferación, como *Myc* y *Ccnd1* (que codifica la CICLINA D1). La inhibición de la actividad cinasa de BCR-ABL1 impide la traslocación

nuclear de la ß-CATENINA y su activación transcripcional (Coluccia et al., 2007). En ratón, la pérdida de *ß-Catenina* conlleva una disminución en la capacidad de auto-renovación en las HSC normales y en las CMLs de CB-LMC, aunque estos efectos aún no han sido demostrados en CMHs y CMLs humanas en un modelo de xenoinjerto (Zhao et al., 2007). Recientemente, se ha demostrado que la inhibición farmacológica de la ß-catenina con indometacina, un modulador de la vía de las prostaglandinas, produce una reducción de CML en un modelo de ratón de LMC (Heidel et al., 2012).

La disminución de la expresión de GSK3ß potencia la autorenovación de los progenitores mediante la activación de ß- CATENINA y el incremento de los niveles de los mediadores de la ruta de Sonic Hedgehog, como por ejemplo, GLI1 y GLI2 (Abrahamsson et al., 2009; Radich et al., 2006). Dos trabajos independientes han puesto de manifiesto la importancia de esta ruta en la autorenovación de los progenitores en CB-LMC. Ambos demuestran que la sobreexpresión de SMO, un activador esencial de esta ruta, potencia el automantenimiento de las CMLs en ratón. De manera inversa, la inhibición de Smo reduce el injerto de las CMLs pero no de las CMHs normales (Dierks et al., 2008; Zhao et al., 2009). Otro trabajo reciente, confirma que la ruta de Sonic Hedgehog es dipensable para la función de las CMHs normales de ratón y sugiere la posibilidad de eliminar progenitores en CB-LMC utilizando inhibidores de SMO, sin dañar las CMHs residuales (Hofmann et al., 2009).

2.2.5) Inhibición de los genes supresores de tumores.

La CB-LMC se caracteriza también por la pérdida de función de genes supresores de tumores. Como se citó en este mismo apartado, las mutaciones inactivantes o deleciones en *p53*, son las responsables de un porcentaje importante de las crisis blásticas mieloides, pero no se detectan en FC-LMC. En los pacientes en CB-LMC mieloide, la pérdida funcional de *p53* también puede ser consecuencia de una degradación incrementada del supresor por mecanismos traduccionales y post-traduccionales dependientes de la expresión de BCR-ABL1, y mediados por HDM2 (Goetz et al., 2001). La inhibición de la actividad tirosín-cinasa de BCR-ABL1 en células que expresan el oncogén, induce la activación de *p53*. Por el contrario, la inactivación de *p53* bloquea la respuesta a imatinib *in vivo* e *in vitro*, aunque la actividad tirosín-cinasa del oncogén esté inhibida. Así que, mutaciones en *p53* pueden contribuir al fenotipo resistente a Imatinib en la CB-LMC (Wendel et al., 2006).

La actividad transcripcional de la fosfatasa y homólogo de tensina (PTEN) está disminuida en CML de LMC en comparación con células madre hematopoyéticas de

donantes sanos (Chen et al., 2009b). PTEN se comporta como un supresor tumoral inhibiendo la supervivencia de las CMLs y el desarrollo de LMC. Se ha comprobado que BCR-ABL1 reprime la expresión de PTEN y que ésta, correlaciona directamente con los niveles de P53. Mientras que la deleción de PTEN aceleraba el desarrollo leucémico, su sobrexpresión conseguía atenuar la leucemia en el modelo CML de ratón de transducción-trasplante (Peng et al., 2010b). Aparte del posible control transcripcional por P53 de PTEN, Chen y col. proponen que la represión de PTEN por parte del BCR-ABL1, se puede deber a que este último, aumenta la expresión de la lipooxigenasa ALOX5 (Chen et al., 2009a), que a su vez se ha demostrado que puede oxidar e inhibir PTEN en líneas celulares de páncreas (Covey et al., 2007). Sin embargo, la hipótesis de que BCR-ABL1 controla indirectamente PTEN a través de ALOX5 está aún por demostrar en LMC (Peng et al., 2010a).

La serina-treonina fosfatasa 2A (PP2A) funciona como un supresor tumoral antagonizando la actividad cinasa de BCR-ABL1 (Neviani et al., 2005). PP2A está inhibida en diversos tipos de cáncer por mecanismos que incluyen la pérdida de expresión/actividad de una o varias de sus subunidades, o el incremento en la expresión de los inhibidores endógenos denominados SET (Iervolino et al., 2002; Li and Damuni, 1998) o CIP2A (Junttila et al., 2007). La supresión de la actividad de PP2A tiene un papel importante en la supervivencia, autorenovación y diferenciación de las células, y por tanto, en el desarrollo de la LMC. La cinasa BCR-ABL1 inhibe PP2A incrementando la expresión de SET (Neviani et al., 2005). A su vez, PP2A es capaz de activar la proteína tirosina fosfatasa 1 (SHP1) que cataliza la defosforilación de BCR-ABL1 y su degradación en el proteosoma (Neviani et al., 2005). Los niveles de proteína PP2A están poco o nada disminuidos en progenitores $CD34^+$ en FC-LMC, pero en CB-LMC son muy bajos y correlacionan inversamente con la expresión/actividad de BCR-ABL1 (Neviani et al., 2007; Neviani et al., 2005). La inhibición de SET y la activación de PP2A son estrategias potenciales para la terapia en LMC. En ese sentido, los activadores químicos de PP2A, como el forskolin o el FTY720 (fingolimod), activan SHP1 y degradan BCR-ABL1 (Liedtke et al., 1998; Neviani et al., 2007; Neviani et al., 2005). Como consecuencia de la reactivación de PP2A se produce la apoptosis de progenitores $CD34^+$ FC-LMC o CB-LMC y se suprime la leucemogénesis *in vivo,* aunque las células sean resistentes a los inhibidores de cinasas (Neviani et al., 2007; Neviani et al., 2005). Finalmente, se ha demostrado que FTY720 también es capaz de suprimir la capacidad de autorenovación y la supervivencia de las CMLs, de una manera independiente de la actividad cinasa de BCR-ABL1, interfiriendo la ruta de la ß-CATENINA. Sin embargo,

las CMHs normales quiescentes no resultaron sensibles a FTY720 (Neviani et al., 2013).

3. La inestabilidad genética es un mecanismo de progresión a la fase blástica de la LMC.

3.1 BCR-ABL1 como inductor de mutaciones en el ADN.

Una de las características más importantes de la CB-LMC es la adquisición de aberraciones cromosómicas y mutaciones adicionales a la aparición de la fusión BCR-ABL1. La frecuencia de alteraciones cromosómicas adicionales durante la FC-LMC es del 7% y aumenta hasta un 40-70% en CB-LMC (Bacher et al., 2005). Sin embargo, como se ha indicado en el apartado anterior, aunque algunas de estas alteraciones moleculares y cromosómicas son más comunes que otras, existe mucha variación entre los pacientes, lo que refleja un estado general de inestabilidad genética, más que un efecto directo sobre un *locus* en particular. Dicho estado es coherente con el fenotipo "mutador" que se le ha asignado a la célula LMC (Burke and Carroll, 2010; Koptyra et al., 2006; Quintas-Cardama and Cortes, 2009). La aparición de estas alteraciones secundarias ha multiplicado el número de trabajos que tratan de explicar cuáles son los mecanismos por los que BCR-ABL1 es capaz de modificar la estabilidad genética celular. Se ha hipotetizado con que la inestabilidad genética en la LMC es responsable de la aparición de mutaciones que confieren resistencia a los TKI, favorecen la aparición de recaídas en los pacientes y facilitan la progresión a las fases más avanzadas, sin duda los tres mayores retos en la terapia de la enfermedad (Skorski, 2008). De hecho, se ha planteado que la inestabilidad se puede originar desde un pequeño compartimento de células madre durante la FC-LMC (Bolton-Gillespie et al., 2013).

La inestabilidad genética en la célula puede surgir como resultado de una respuesta aberrante al daño incrementado en el ADN. Dicha respuesta consiste en alteraciones en los sistemas de reparación y/o fallos en los mecanismos de vigilancia genómica. En la LMC, estos mecanismos son modulados directamente por la actividad cinasa de BCR-ABL1 (Figura 9) o pueden ser, en algunos casos, independientes de esa actividad.

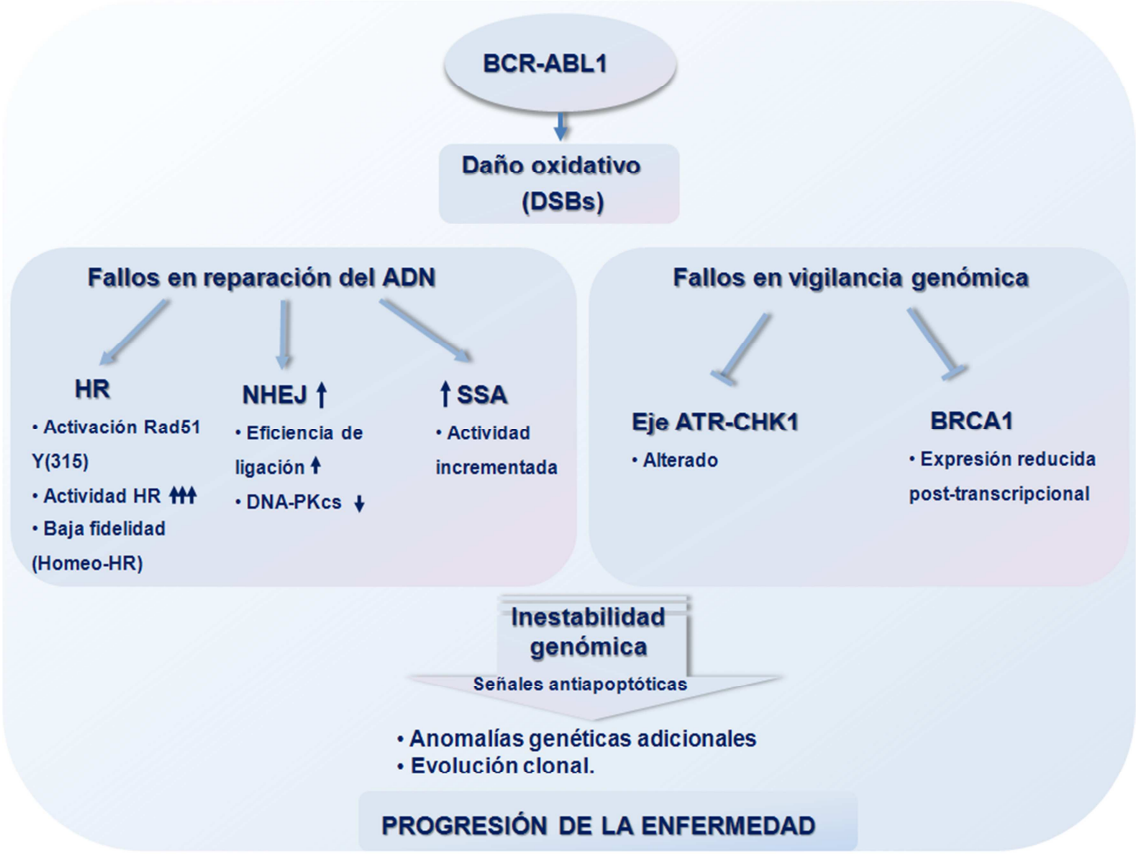

Figura 9: Alteraciones en la respuesta al daño en el ADN en la LMC.

3.1.1 Incremento del daño en el ADN

Se han descrito mecanismos diferentes, por los que BCR-ABL1 puede generar daño en el ADN de una célula. El más estudiado, consiste en que BCR-ABL1 induce la producción de especies reactivas de oxigeno (ROS, del inglés *Reactive Oxigen Species*) en la célula, causando daño oxidativo y mutagénesis (Koptyra et al., 2006; Sattler et al., 2000). Las ROS consisten en aniones de radicales superóxido que se transforman en peróxido de hidrógeno y radicales hidroxilo. Estudios en líneas celulares transformadas con BCR-ABL1 y en $CD34^+$ LMC han demostrado que éstas contienen entre 2-6 veces más ROS que sus controles normales (Cramer et al., 2008; Koptyra et al., 2006; Nowicki et al., 2004). Los mecanismos de producción endógena de ROS en células LMC sólo se están empezando a esclarecer. Kim y col. (Kim et al., 2005), demostraron que la activación de la ruta PI3K/mTOR por BCR-ABL1 aumenta los niveles de ROS mitocondriales. En otro estudio independiente, se han identificado las oxidasas NADPH, fundamentalmente NOX-4, como la fuente principal de generación de ROS (Naughton et al., 2009), o recientemente, la participación de la RAC2 GTPasa en la alteración del potencial de membrana y el flujo electrónico mitocondrial a través de MRC-cIII, y por tanto, en la generación de altos niveles de

estas moléculas reactivas en CMHs y progenitores de LMC (Nieborowska-Skorska et al., 2012). Las ROS pueden causar daño en todos los residuos de desoxiribosa e incluso en nucleótidos libres. El resultado es la generación de nucleobases oxidadas, como por ejemplo 8-oxodG, y roturas de doble hebra en el ADN (DSB del inglés *Double Strand Break*) (Beckman and Ames, 1997). El número de bases oxidadas en el ADN en una célula normalmente es 10^4 por día, y se generan unas 50 DSBs por célula y por ciclo. Las células de LMC presentan entre 3 y 8 veces más nucleobases oxidadas y entre 4 y 8 veces más DSBs (Cramer et al., 2008; Koptyra et al., 2006; Nowicki et al., 2004).

El daño en el ADN también se puede producir como consecuencia de las radiaciones ionizantes y los fármacos genotóxicos que se usan como parte de la terapia de los pacientes con LMC. Las células que expresan BCR-ABL1 acumulan muchas más lesiones inducidas por irradiación y quimioterapia en comparación con sus controles, lo que se traduce en un aumento de las alteraciones cromosómicas (Dierov et al., 2009; Koptyra et al., 2008).

Se ha observado que la enzima citidina-deaminasa inducida por activación específica de células B (AID), se sobreexpresa en blastos linfoides LMC pero no en blastos mieloides. BCR-ABL1 aumenta la expresión de la enzima que contribuye a la aparición de mutaciones adicionales en el ADN y esas mutaciones ocurren también en el mismo dominio cinasa del oncogén (Klemm et al., 2009). Sin embargo, en adultos la incidencia de CB-LMC linfoide es baja, por lo que este mecanismo no justifica la aparición de inestabilidad genética en la mayoría de las CB-LMC mieloides. De forma alternativa, se ha propuesto que el daño en el ADN es consecuencia de la proliferación incontrolada de las células que expresan BCR-ABL1, pues las enzimas polimerasas podrían causar mayor número de errores durante el proceso replicativo (Kunkel and Bebenek, 2000), o que la expresión de BCR-ABL1 en las células está asociada a un incremento en la expresión de polimerasas de baja fidelidad, como la ADN polimerasa ß (Canitrot et al., 1999).

3.1.2 Alteraciones en las principales rutas celulares de reparación en LMC.

Los efectos de BCR-ABL1 sobre los distintos mecanismos de reparación se resumen en la figura 10.

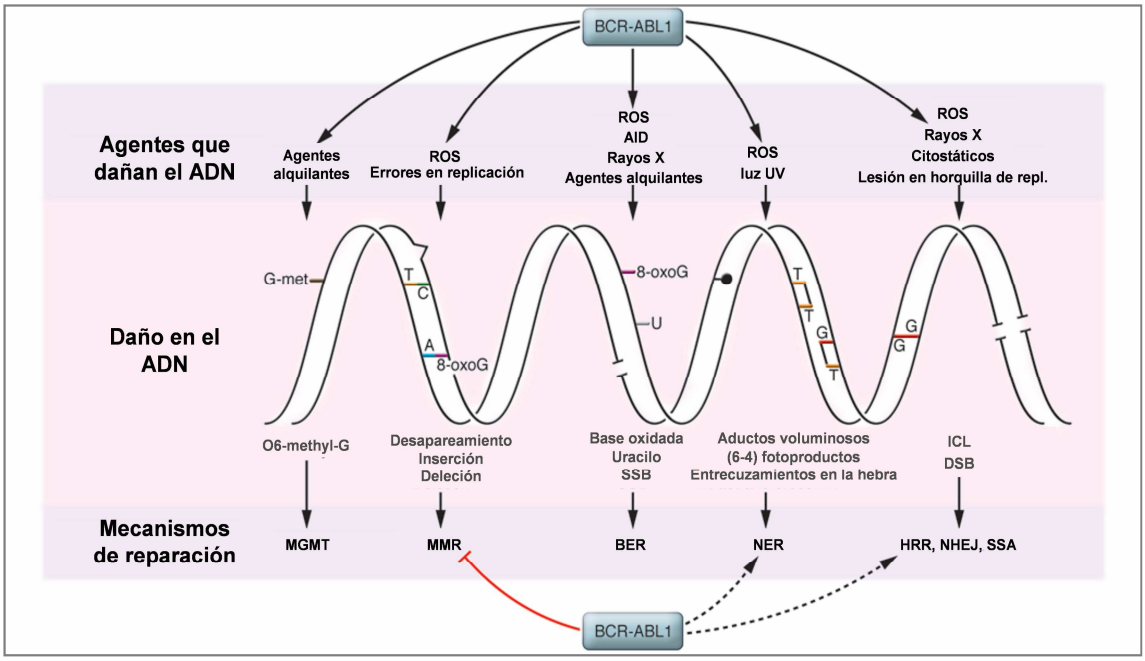

Figura 10. BCR-ABL1 induce distintos tipos de daño y regula los principales mecanismos de reparación del ADN. Tomado y modificado de Perroti y col. (Perrotti et al., 2010).

El daño en el ADN puede reflejarse como la alteración en un único nucleótido, la rotura en una de las dos hebras del ADN (SSB, del inglés *Single Strand Break*) o una DSB. Las alteraciones en un único nucleótido se reparan a través del mecanismo de reparación de desapareamiento de bases (MMR, del inglés *Mismatch Repair*) o por el mecanismo de escisión de nucleótidos (NER del inglés *Nucleotide Excision Repair*) (Figura 11).

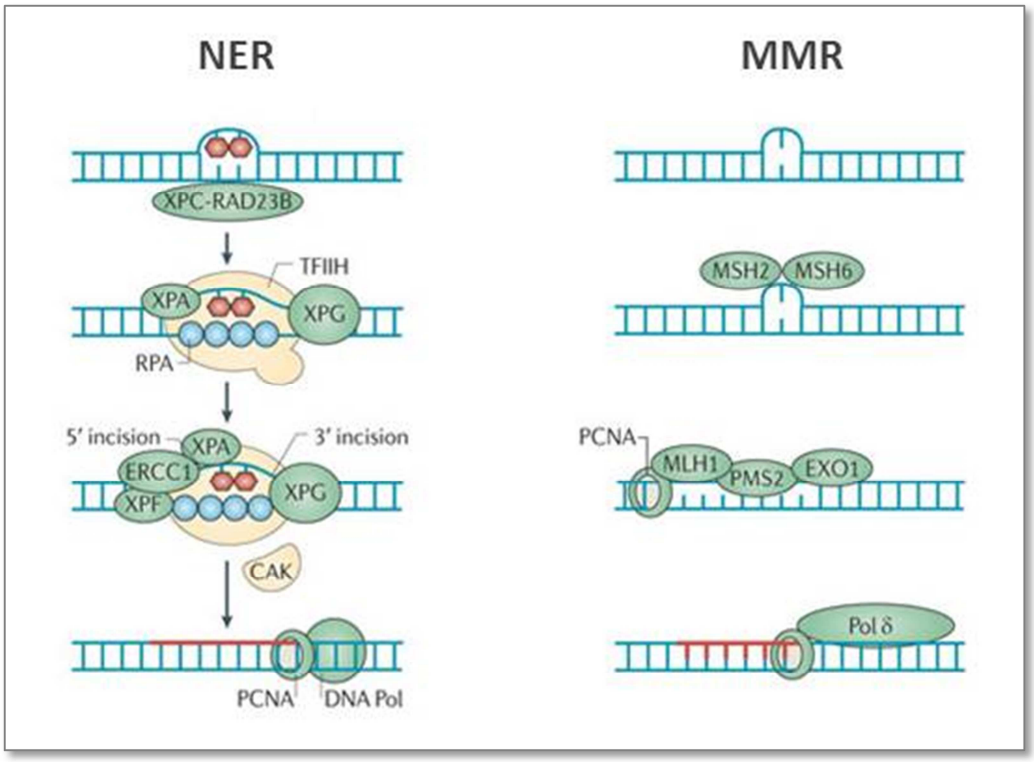

Figura 11: Mecanismos de reparación de la célula para alteraciones en un único nucleótido. Mecanismo de escisión de nucleótidos (NER) y mecanismo de reparación de desapareamiento de bases (MMR).Tomado de Lange y col. (Lange et al., 2011).

Las roturas de hebra se reparan por recombinación homóloga (HR, del inglés *Homologous Recombination Repair*), un mecanismo fidedigno que puede producirse cuando una cromátida hermana está disponible como molde; es decir, en las fases S y G2 del ciclo celular. Alternativamente, puede repararse por el mecanismo de unión de extremos no homólogos (NHEJ, del inglés *Non Homologous End-Joining*). El NHEJ es el mecanismo predominante en mamíferos para la reparación de DSBs, pero es propenso a errores, ya que introduce pequeñas deleciones o inserciones en las hebras reparadas. Las mutaciones en el ADN pueden ocurrir porque existan alteraciones en los genes o proteínas de las rutas NER o MMR, alteraciones en las proteínas que reconocen y reparan las DSBs y/o desbalance hacia procesos de reparación no fidedignos de DSBs, o cuando se producen fallos en los reguladores del ciclo celular, permitiéndose la replicación del ADN dañado. Esto último puede deberse a defectos en los sensores de daño o alteraciones en las proteínas necesarias para detener el ciclo celular (Burke and Carroll, 2010).

Entre el 50-90% de los pacientes resistentes a imatinib y un 23% de pacientes no tratados presentan mutaciones puntuales en el dominio cinasa de *BCR-ABL1* (Willis et al., 2005). Los mutantes de *BCR-ABL1* resistentes a los inhibidores de cinasas

presentan alteraciones en la actividad cinasa, en la utilización del sustrato y en la potencia de transformación (Griswold et al., 2006; Willis et al., 2005). Estas alteraciones tienen un impacto en la progresión de la enfermedad. De hecho, las mutaciones puntuales de *BCR-ABL1* se asocian con una mayor probabilidad de transición a la fase blástica, lo que sugiere una inestabilidad genética aumentada en esas células (Soverini et al., 2005). No se conoce en la actualidad qué impacto tienen las mutaciones puntuales en el resto del genoma en pacientes que progresan a la CB-LMC.

3.1.2.1.1) Reparación de desapareamientos de bases (MMR).

MMR repara los desapareamientos de base, cuando una o pocas bases se incorporan incorrectamente o mutadas en el ADN. En eucariotas, los desapareamientos son reconocidos por MutSß (heterodímero MSH2-MSH3) o MutSα (heterodímero MSH2-MSH6). El complejo MutL1 (heterodímero MLH1-PMS2) realiza una incisión en una de las hebras y la degrada hasta superar el desapareamiento, luego ese espacio se vuelve a rellenar con las bases apropiadas por la polimerasa δ (Martin et al., 2010) (Figura 11). El efecto de BCR-ABL1 sobre el MMR sólo se ha analizado en un trabajo en el que se concluye que, tanto en progenitores hematopoyéticos de ratón que expresan BCR-ABL1 como en células $CD34^+$ de pacientes con LMC, la actividad MMR está disminuida con respecto a sus controles (Stoklosa et al., 2008). La expresión de PMS2 y MLH1 también estaba disminuida en estos dos modelos celulares, aunque estas diferencias sólo eran detectables con técnicas de inmunofluorescencia. Imatinib revirtió la actividad MMR a sus niveles normales, lo que indica que el efecto estaba mediado por la actividad tirosina cinasa del oncogén (Stoklosa et al., 2008).

3.1.2.1.2) Reparación por escisión de nucleótidos (NER).

NER es la ruta que se encarga de la reparación del daño inducido por la radiación ultravioleta (UV) en el ADN, como los dímeros de pirimidina y otros fotoproductos. También se encarga de corregir algunas distorsiones de la hélice de ADN que son voluminosas. Las lesiones por UV se reconocen por el heterodímero formado por las proteínas de unión a ADN dañado (DDB 1 y 2). Las distorsiones de hélice son reconocidas por el complejo formado por la proteína del *Xeroderma pigmentosum* grupo C (XPC) junto a RAD23B. Ambos complejos reclutan otras proteínas NER para la reparación, como el factor de transcripción TFIIH (compuesto por XPB, XPD, p62, p52, p44 y p34), que tiene actividad helicasa. También son reclutadas XPG y XPF, que generan las incisiones en el ADN donde se une PCNA y las DNA polimerasas δ y ε que rellenan los espacios para concluir el proceso (Sertic et al., 2012) (Figura 11).

Existen varios trabajos que han estudiado la interacción de BCR-ABL1 con la vía NER, donde se muestran algunos resultados contradictorios.

El estudio pionero en el campo mostraba en levaduras que el dominio CDC24 de BCR-ABL1 tenía capacidad para unirse a la proteína XPB, y que esa interacción era independiente de la actividad cinasa de BCR-ABL1 (Takeda et al., 1999). Esta interacción no fue reproducible en líneas celulares humanas Ph^+. Más tarde se sugirió que el efecto descrito en NER era el resultado de la interferencia en la formación del complejo TFIIH, por un mero impedimento estérico inducido cuando BCR-ABL1 se unía a XPB (Maru et al., 2001). Este efecto sólo se producía a niveles suprafisiológicos de expresión de BCR-ABL1 *in vitro*. Varios grupos han mostrado que la expresión de BCR-ABL1 sensibiliza a las células a la radiación UV y al daño que induce el cisplatino (Laurent et al., 2003; Maru et al., 1999; Takeda et al., 1999). En este sentido, la línea celular 27-1 tiene un defecto en la vía NER corregible por XPB. La expresión de BCR-ABL1 en la línea celular impedía a XPB corregir este defecto en la reparación (Maru et al., 2001).

Canitrot y colaboradores, en el año 2003 (Canitrot et al., 2003), introducen la idea de que el efecto de BCR-ABL1 sobre la vía NER puede ser dependiente del tipo celular estudiado. Así, en la línea celular pro-B BaF3, BCR-ABL1 disminuía dos veces la actividad NER y sensibilizaba a las células a la radiación UV, de acuerdo con los anteriores estudios. En todo el resto de líneas celulares mieloides estudiadas, la actividad NER aumentaba dos veces y se hacían resistentes a la radiación UV. Todos estos efectos eran reversibles por imatinib, luego dependían de la actividad cinasa. Un trabajo posterior corroboró en diferentes líneas transformadas con BCR-ABL1, como MO7E y 4A2+, la resistencia a UV de estas células. En este trabajo, se detectan menor número de dímeros de pirimidina ciclobutano pero mayor número de DSBs en las células que expresan BCR-ABL1. Por tanto, sugieren que el oncogén podría estar aumentando la velocidad de la reparación de las lesiones inducidas por UV, abriendo la posibilidad de que la reparación no fuera del todo fidedigna (Laurent et al., 2003). En este sentido también, Sliwinski y colaboradores, mostraron una actividad NER incrementada y resistencia a UV tanto en líneas celulares mieloides como en celulas primarias de médula osea de ratón. El tratamiento con imatinib en este caso también revirtió el efecto descrito (Sliwinski et al., 2008). La aparición del polimorfismo (499C-939) en el análisis de haplotipo del gen XPC se ha asociado a una mejor respuesta a imatinib en pacientes en FC-LMC (Guillem et al., 2010). Finalmente, aunque los efectos de BCR-ABL1 sobre NER se han documentado en líneas celulares y se ha

impuesto el concepto de que el oncogén está estimulando esta ruta de reparación, el significado fisiológico en la progresión a la CB-LMC permanece aún por dilucidar.

3.1.2.2) Reparación de roturas de la doble hebra (DSB).

Las DSBs son las lesiones en el ADN más mutágenicas porque su reparación resulta más problemática. Una unión errónea de extremos libres en el ADN puede causar directamente la aparición de una translocación en la célula. En el caso de las células de LMC, se demostró que éstas adquieren mayor cantidad de translocaciones y eventos aneuploides que sus controles después de recibir un daño exógeno en el ADN (Burke and Carroll, 2010). Existe suficiente evidencia científica que indica que NHEJ y HR, las rutas más importantes en la reparación de DSB en células de mamíferos están alteradas en las células de LMC (Figura 12).

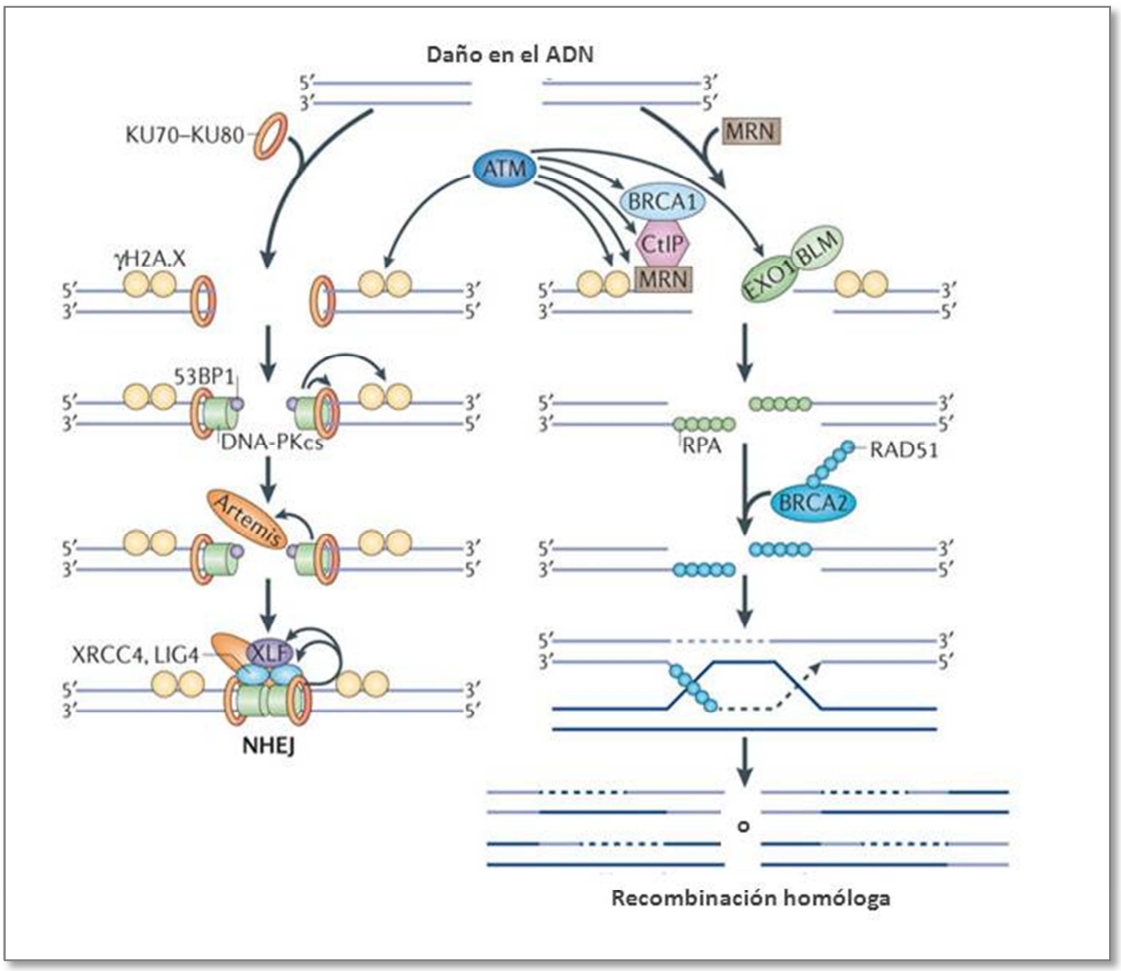

Figura 12: Reparación de roturas de doble hebra. Esquema de las principales rutas de reparación de DSBs: NHEJ y HR (Chowdhury et al., 2013).

3.1.2.2.1) Reparación por unión de extremos no homólogos (NHEJ).

El NHEJ es un mecanismo rápido de reparación que ocurre en la célula tras producirse una doble rotura en el ADN. No necesita una hebra molde homóloga por lo que es la ruta preferente cuando las células están en la fase G0 y G1 del ciclo celular. La ruta clásica o canónica de NHEJ, implica la unión y el reconocimento de los extremos de la DSB por el heterodímero Ku70/Ku80 y el reclutamiento de la nucleasa DNA-PKcs-Artemis. Los pasos de ligación posteriores están dirigidos por el complejo ADN ligasa IV y XRCC4, que está estimulado por XLF/CERNUNNOS (Figura 12). NHEJ es un mecanismo de reparación no fidedigno, porque en muchas de las roturas reparadas se producen pequeñas deleciones o inserciones de 1 a 4 nucleótidos (indels) e incluso translocaciones cuando los cromosomas se yuxtaponen de forma incorrecta (Davis and Chen, 2013).

Slupianek y colaboradores (Slupianek et al., 2006), usando un ensayo de fluorescencia, determinaron que la actividad NHEJ para reparar extremos romos estaba aumentada al doble en la línea celular 32Dcl3 transformada con BCR-ABL1, comparada con su parental. La actividad NHEJ se cuadruplicaba si en la unión participaban extremos 5' cohesivos. La fidelidad de la reparación también se veía comprometida, porque se detectaba un aumento de inserciones pequeñas y deleciones de gran tamaño cuando estaba presente BCR-ABL1. Gaymes y colaboradores (Gaymes et al., 2002), corroboraron estos resultados en células de pacientes LMC y células K562, donde la eficiencia de ligación de extremos se incrementaba entre tres y cinco veces, comparado con células $CD34^+$ normales. Este efecto iba acompañado de un aumento en la frecuencia de errores, que incluía la aparición de deleciones de entre 30-400 pb. La incubación de las células con anticuerpos contra Ku70 y Ku80 restauró la frecuencia y el tamaño de las deleciones a niveles de los controles. Esto no se produjo cuando se utilizaron anticuerpos contra DNA-PKcs, resaltando la importancia del heterodímero en el efecto. Estos resultados apoyan el concepto de que la expresión de BCR-ABL1 incrementa la reparación no fidedigna de las DSB en el ADN a través del mecanismo de NHEJ.

Sin embargo, Deutsch y colaboradores (Deutsch et al., 2001) mostraron que los niveles de expresión de la subunidad catalítica de proteína cinasa dependiente de ADN (DNA-PKcs) estaban disminuidos en células $CD34^+$ LMC con respecto a sus controles. Los niveles de DNA-PKcs eran inversamente proporcionales a los de BCR-ABL1 en líneas celulares y prácticamente indetectables en células primarias de LMC. La supresión de DNA-PKcs estaba mediada directamente por la actividad cinasa del

oncogén de forma post-traduccional, y podía revertirse mediante inhibidores del proteosoma. Este efecto implica que la supresión de DNA-PKcs estaba regulada por la ruta de degradación ubiquitina-proteosoma. En este trabajo no se detectaron diferencias en la expresión de los componentes del heterodímero Ku70 y Ku80. Es motivo de discusión que en las células que expresan BCR-ABL1 aumente la actividad de NHEJ, cuando es inexistente la expresión de la proteína que produce la cátalisis principal de la ruta, la DNA-PKcs.

Existe un segundo mecanismo alternativo de NHEJ que es mucho más propenso a introducir errores en el ADN de la célula. En este mecanismo (b-NHEJ, de *back-up*), la DNA ligasa IIIα puede sustituir a la DNA ligasa IV, si no está disponible o no es reclutable. También la poli-ADP-ribosa polimerasa (PARP) tiene un papel en el b-NHEJ, como se ha demostrado en células deficientes en Ku70 y Ku80. Las proteínas del b-NHEJ producen un aumento de errores en los productos de reparación como consecuencia de una ligación de micro-homología. Sallmyr y colaboradores han estudiado el papel de estas proteínas que participan en el b-NHEJ, para reparar DSB en LMC (Sallmyr et al., 2008). Las líneas celulares de LMC expresan niveles inferiores de DNA ligasa IV y Artemis, pero niveles muy aumentados de DNA ligasa IIIα y proteína de Werner (WRN). La misma sobreexpresión se observó en muestras primarias de pacientes LMC, y ésta correlacionaba directamente con los niveles de BCR-ABL1. No se observaron cambios en la expresión de Ku80, DNA-PKcs, XRCC4, XRCC1 o PARP (Sallmyr et al., 2008). La expresión de ARN de interferencia contra WRN y DNA ligasa IIIα producía, en líneas celulares de LMC, un aumento de DSBs tanto basales como inducidas por irradiación, así como una disminución en la eficiencia de unión de los extremos. La sobreexpresión de Artemis en estas células disminuía el tamaño de las deleciones en el DNA producidas en las DSB (Sallmyr et al., 2008). Para mayor controversia, Chakraboty y col. han demostrado recientemente que las células $CD34^+$ de pacientes de LMC contienen mayor inestabilidad cromosómica después de someterlas a daño con irradiación. Curiosamente, proponen que la inestabilidad ocurre a consecuencia de un mecanismo cíclico de rotura-fusión-puente en el ADN, que estaría mediado por un incremento en la actividad de NHEJ clásica. Este fenotipo correlacionó con el aumento en los niveles de DNA-PKcs en las células de pacientes de LMC y fue revertido con Dasatinib (Chakraborty et al., 2012). Todos estos datos parecen indicar que BCR-ABL1 podría estar activando preferentemente la actividad del NHEJ clásico ó probablemente, del b-NHEJ, aumentando la actividad NHEJ total pero disminuyendo la fidelidad del mecanismo de reparación.

3.1.2.2.2) Reparación por recombinación homóloga (HR).

HR es un proceso complejo que implica la utilización de una cromátida hermana para la reparación de la DSB, con mucha más fidelidad que NHEJ. Por tanto, sólo ocurre cuando las cromátidas hermanas están presentes, durante la fase S o G2 del ciclo celular. Brevemente, La HR se activa por la señal de daño en el ADN que depende de serina/treonina cinasa ATM y el complejo MRN (MRE11-RAD50-NBS1) que, junto con la proteína CtIP y la exonucleasa Exo1 producen la resección que procesa el extremo de la DSB, generando extremos protuberantes 3' de ADN de una única cadena (ssDNA). Posteriormente, el extremo de cadena simple se proteje mediante la proteína RPA, que forma el sustrato para que se una al ADN la recombinasa RAD51. La recombinasa cataliza la invasión de la hebra de ADN simple sobre la cromátida hermana. Usando el ADN de la cromátida hermana como molde, el ssDNA se elonga y se forman las uniones de Holliday entre dichas cromátidas. Finalmente, las uniones de Holliday se resuelven y los extremos de ADN se ligan de forma libre de errores. La familia de helicasas RecQ (BLM, WRN, RTS, RecQL1, RecQL5), se encarga de desenrollar el ADN durante la HR tanto en la iniciación, como al final, en la resolución de los intermediarios (West, 2003) (Figura 12).

Todos los trabajos que han relacionado la expresión de BCR-ABL1 con efectores de la HR han sido realizados por componentes de un único grupo, el del Dr. Tomasz Skorski en la Universidad de Temple (Filadelfia). Las células que expresan BCR-ABL1 presentan una eficiencia de HR aumentada, medida por el ensayo de expresión de ISce-I (Nowicki et al., 2004; Slupianek et al., 2002; Slupianek et al., 2006), pero no fidedigna (Nowicki et al., 2004; Slupianek et al., 2006). Aunque el número de eventos de reparación era muy similar, la frecuencia de mutación en términos de sustituciones de base única estaba cien veces aumentada en las células BCR-ABL1 con respecto a sus controles. Esas sustituciones, sin embargo, no se agrupaban en torno a la DSB. Se ha podido calcular que la tasa de mutación general de HR en células que expresan BCR-ABL1, es de $6x10^{-3}$, donde el 55% de las mutaciones son transiciones G/C a A/T o transversiones G/C a T/A. Por el contrario, la HR no introduce errores en las células control (Nowicki et al., 2004).

El efecto de BCR-ABL1 sobre RAD51, la recombinasa clave en el proceso, se ha puesto de manifiesto en varios trabajos. Slupianek y colaboradores, demostraron que BCR-ABL1 afecta a la expresión de RAD51, regulando su transcripción, degradación y activación (Slupianek et al., 2001). El aumento transcripcional de RAD51, está mediado por la activación de STAT5 vía BCR-ABL1. El oncogén bloquea la

degradación de RAD51 impidiendo la activación de la CASPASA 3. La activación de RAD51 se produce por la fosforilación directa de BCR-ABL1 sobre su tirosina Y315 (Slupianek et al., 2001), que se induce por la interacción del dominio SH3 del oncogén sobre las regiones ricas en prolina de RAD51 (Slupianek et al., 2011a). El resultado de esa fosforilación es el aumento en la formación de *foci* nucleares de RAD51 en los sitios de recombinación y un fenotipo resistente a drogas citotóxicas, como MMC y cisplatino (Slupianek et al., 2011a; Slupianek et al., 2001). En este sentido, se ha comprobado que BCR-ABL1 induce una reparación por recombinación homóloga no fidedigna (HomeoRR) al fosforilar RAD51 en Y315. El diseño de péptidos que bloquean esta interacción consiguen disminuir la formación de *foci* de RAD51 e inhibir la HomeoRR en células BCR-ABL1 (Slupianek et al., 2011a). El grupo del Tomasz Skorski ha sugerido además, que esta fosforilación podría estimular la HR potenciando la formación de complejos RAD51/RAD52 (Chen et al., 1999). BCR-ABL1 es también capaz de fosforilar y activar otros parálogos de RAD51, como RAD51B (Slupianek et al., 2009).

La proteína de Bloom (BLM) es otra de las proteínas de HR analizadas en LMC. Se ha observado que la expresión de BCR-ABL1 es suficiente para aumentar la expresión y la actividad helicasa de Bloom, a niveles similares a los que se consiguen en células control cuando se estimulan con factores de crecimiento (Slupianek et al., 2005). BCR-ABL1 también es capaz de aumentar la expresión y la activación de NBS1 (Rink et al., 2007), que es uno de los componentes del complejo de resección MRN, así como la expresión y la localización en el núcleo de la proteína mutada en el síndrome de Werner (WRN) (Slupianek et al., 2011b), que participa en el procesamiento de la DSB.

3.1.2.2.3) Reparación por alineamiento de cadena simple (SSA).

El SSA (del inglés *Single Strand Annealing*) es un proceso no conservativo de HR, que puede reparar las DSBs que tienen lugar entre dos repeticiones idénticas en la secuencia de ADN. Este proceso es muy poco fidedigno, porque durante su desarrollo se produce la deleción de la secuencia que se encuentran entre las repeticiones y también de una de las repeticiones que participa en el proceso. Muchas de las proteínas que lo componen también participan en otros mecanismos de reparación de DSBs, por tanto, no es un proceso totalmente independiente. Una proporción importante de las proteínas que componen el SSA y HR son comunes, con la excepción de RAD51 que no participa en el SSA. Tres trabajos han analizado el efecto de BCR-ABL1 sobre el SSA (Cramer et al., 2008; Fernandes et al., 2009; Salles et al., 2011). Todos ellos determinaron que la actividad SSA se incrementaba en función del

aumento en la expresión de BCR-ABL1, siendo este reversible por imatinib (Fernandes et al., 2009). No se observaron efectos en los niveles de expresión de proteínas clave en SSA, como ERCC1 o RAD52, pero sí un incremento en su co-localización en células que expresaban la oncoproteína (Fernandes et al., 2009). Salles y col. detectaron un aumento de la actividad SSA y del NHEJ en líneas celulares de LMC. Además, demostraron que BCR-ABL1 induce la acumulación de la proteína CtIP en respuesta irradiación y esto se traduce en un aumento en la actividad de resección de los extremos de la DSB en las células. El tratamiento con imatinib eliminaba la acumulación de CtIP y la inhibición de CtIP en la célula revertía el aumento de la actividad SSA (Salles et al., 2011).

El análisis global de todos los mecanismos de reparación en el ADN en LMC indica que las células que expresan BCR-ABL1 presentan una actividad disminuida de MMR, lo que facilita la introducción de mutaciones puntuales. La expresión del oncogén induce una ruta NER que resulta mutagénica, y estimula todos los mecanismos de reparación de DSB (NHEJ, HR y SSA), comprometiendo a la vez su fidelidad. El resultado es la introducción en el ADN de mutaciones puntuales mediante HR, pérdida de nucleobases asociada a NHEJ y aparición de grandes deleciones debido a la actividad SSA (Perrotti et al.2010). En conjunto, la generación de ROS en combinación con una regulación alterada de estos mecanismos, contribuye al fenotipo mutador que genera la inestabilidad genética en la célula de LMC, responsable de la progresión de la FC-LMC a la CB-LMC (Quintas-Cardama and Cortes, 2009).

3.2 Fallos en la vigilancia del genoma. Influencia de BCR-ABL1 en la regulación de los sensores/mediadores del daño en el ADN.

Una hipótesis alternativa, pero no excluyente, al efecto directo de BCR-ABL1 sobre los mecanismos de reparación, consiste en que la oncoproteína podría estar alterando los mecanismos celulares que reconocen y responden al daño en el ADN. En eucariotas, esta respuesta es muy compleja y está mediada sobre todo por dos proteínas nucleares que pertenecen a la familia de las fosfatidil-inositol cinasas. Una es la proteína mutada de la Ataxia telangiectasia (ATM) y otra su homólogo, la proteína relacionada con RAD3 y de la Ataxia telangiectasia (ATR). ATM puede fosforilar numerosos sustratos pero cabe destacar entre ellos a CHK2, p53 y BRCA1. ATM puede interaccionar y fosforilar a c-ABL1 después del daño con irradiación, por lo que está implicado en la respuesta de la tirosina cinasa no oncogénica (Baskaran et al., 1997). ATR también fosforila un amplio elenco de sustratos, donde destaca la

fosforilación de CHK1. No se han encontrado mutaciones o deleciones en *ATM*, ni en líneas celulares ni en células de pacientes LMC (Melo et al., 2001). Aunque ATM inmunoprecipita con BCR-ABL1, tampoco se han encontrado diferencias en cuanto a su capacidad para fosforilar a CHK2 o p53 en las células que expresan el oncogén (Dierov et al., 2004), por lo que no parece que ATM tenga un papel importante en la transición a la CB-LMC. No ocurre lo mismo con su sustrato BRCA1.

El supresor de tumores BRCA1 participa en multitud de funciones celulares. Forma parte del complejo asociado a la vigilancia genómica (BASC) (Wang et al., 2000) y, entre otras, tiene una función bien definida preservando la integridad genética, porque organiza los sensores de daño y participa en la reparación de las DSBs, promoviendo la HR (Narod and Foulkes, 2004) (Figura 13).

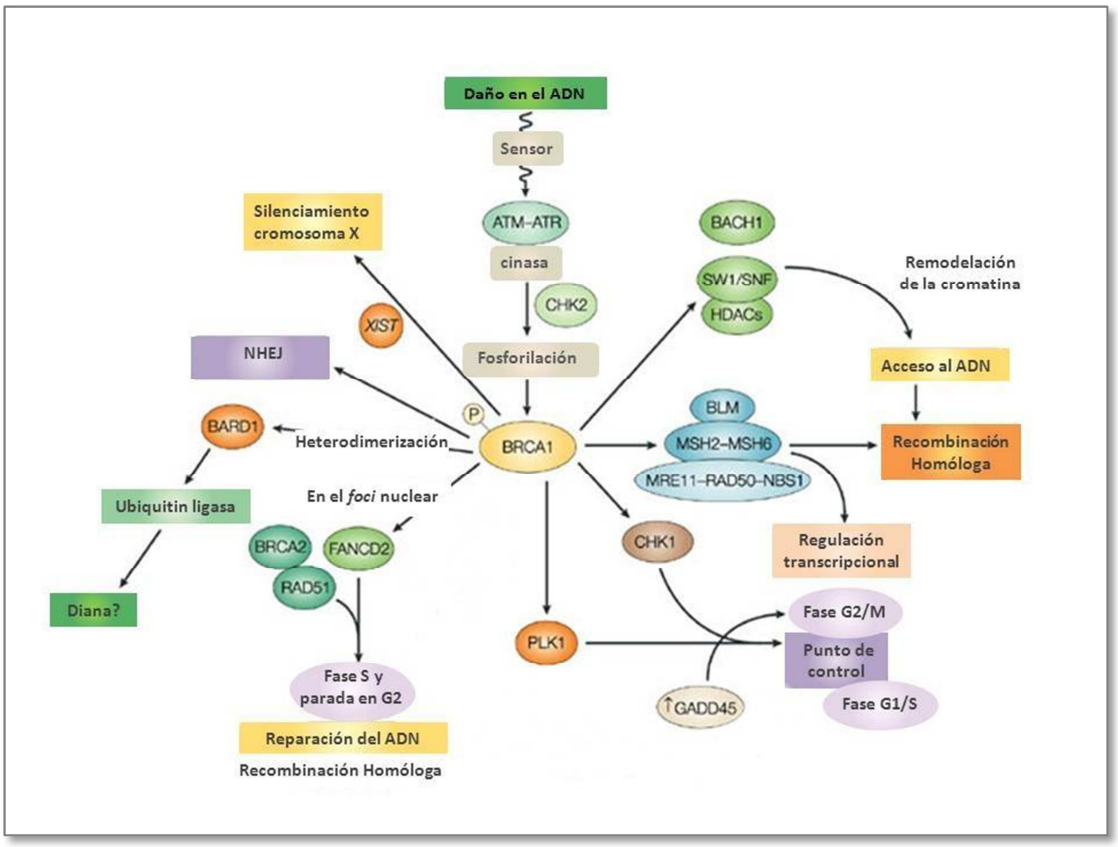

Figura 13: Principales funciones del supresor de tumores BRCA1 (Narod and Foulkes, 2004).

Deutsch y colaboradores demostraron en líneas celulares transformadas y en células mononucleadas de pacientes CML, que la expresión de BRCA1 era indetectable (Deutsch et al., 2003). Más aún, la expresión de BRCA1 correlacionaba de forma inversa con la expresión del oncogén en una línea celular con expresión de BCR-ABL1

inducible. La desaparición de BRCA1 dependía de la actividad cinasa de la oncoproteína y su regulación ocurría a nivel post-traduccional, porque los niveles de BRCA1 se revertían con lactacistina, un inhibidor del proteosoma. Curiosamente, en el mismo trabajo se detectó una mayor frecuencia de entrecruzamiento de cromátidas hermanas en las células LMC, que es reflejo de una actividad HR incrementada, aún a pesar de la falta de expresión de BRCA1 en las células. Recientemente, otro trabajo ha confirmado la disminución de BRCA1 en células que expresan BCR-ABL1 en diferentes dosis. Los autores demostraron que la eliminación de BRCA1 mediada por la oncoproteína era suficiente para afectar la función del control de ciclo en mitosis e inducir divisiones aberrantes y resistencia a Nocodazol (Wolanin et al., 2010). Una de las nuevas funciones descubiertas recientemente de BRCA1 está directamente relacionada con la elección de la ruta de reparación que va a suceder en la célula cuando se produce la DSB. Durante el procesamiento del extremo de la DSB, la proteína 53BP1 se posiciona atrayendo los efectores para favorecer el NHEJ, bloqueando la resección del extremo y por tanto, la iniciación de HR durante las fase G1 del ciclo celular. Sin embargo durante la fase S, BRCA1 junto con el factor CtIP antagonizan el efecto del complejo 53BP1-RIF1, degradan 53BP1 del extremo que queda disponible para que el complejo MNR inicie la resección de una de las hebras y se dispare la HR (Figura 14). En células de ratón deficientes para *Brca1* se ha demostrado que 53BP1 bloquea la resección del extremo, provocando un NHEJ tóxico que es el responsable de la aparición de inestabilidad cromosómica. La depleción de *53bp1* en estas células era capaz de revertir el fenotipo cromosómico inestable (Bouwman et al., 2010; Bunting et al., 2012; Bunting et al., 2010; Cao et al., 2009; Escribano-Diaz et al., 2013).

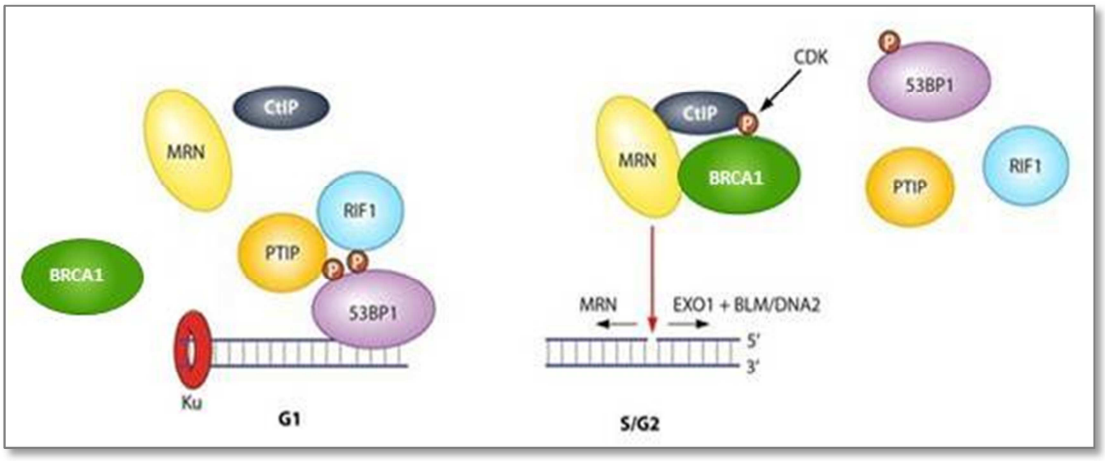

Figura 14: Modelo de competición en la reparación de DSB en fase G1 y S/G2. Modificado de Daley y col. (Daley and Sung, 2014).

De la misma forma que ATM, ATR se asocia a BCR-ABL1 tras el daño al ADN. Dierov y colaboradores, propusieron que la señalización de ATR está inhibida en células LMC (Dierov et al., 2004). La fosforilación de CHK1 estaba reducida y por tanto, su función, el control de ciclo en la fase S. La eliminación del control de ciclo induce una replicación inapropiada a pesar de la presencia del daño en el ADN, lo que desencadenaría una acumulación de lesiones, incluidas las DSBs. Nieborowska-Skorska y colaboradores, concluyeron más tarde, que el eje ATR/CHK1 se encuentra estimulado en células LMC, donde CHK1 podría activarse por su fosforilación en la S345, inducir la parada de ciclo y facilitar la reparación de las DSBs (Nieborowska-Skorska et al., 2006). BRCA1 tiene como sustrato también a CHK1 y a su vez es fosforilado por ATR y por la cinasa AKT (Altiok et al., 1999). Sin embargo, aunque la posible alteración del eje ATR-BRCA1-CHK1 mediada por la interferencia de AKT no ha sido estudiada en LMC, sí se ha demostrado en líneas de cáncer de mama y ovario (Guirouilh-Barbat et al., 2010; Plo et al., 2008; Tonic et al., 2010). Parece evidente que resultan necesarios más estudios para resolver esta controversia, pero en líneas generales se acepta que BCR-ABL1 altera la respuesta celular normal al daño en el ADN, a través de su interacción con ATR.

3.3. Hipertrofia centrosómica.

Los centrosomas son orgánulos vitales para el funcionamiento de la maquinaria mitótica. Se encargan de organizar el huso mitótico para asegurar la separación bipolar de los cromosomas. Ganem y col. han demostrado que el mecanismo por el que se asocian los extra-centrosomas y la aparición de la inestabilidad cromosómica no se debe a la formación durante la mitosis de anafases multipolares. Es más, describen que los errores en la segregación de los cromosomas están causados por la formación de una forma intermedia de huso acromático multipolar durante la mitosis en la que los microtúbulos se asocian con cinetocoros merotélicos (Ganem et al., 2009) (un cinetocoro se conecta a más de un polo mitótico). Por esta razón, las alteraciones en los centrosomas pueden causar directamente alteraciones cromosómicas y aneuploidía, características comunes en la CB-LMC. Además del papel estructural descrito, los centrosomas tienen importancia como reguladores de la entrada a mitosis porque se asocian con p53 (Tritarelli et al., 2004). Las alteraciones en el número y tamaño de los centrosomas son muy frecuentes en todos los tipos de cáncer y correlacionan con la progresión maligna de la enfermedad (Fukasawa, 2005). Dos grupos distintos han investigado los defectos centrosómicos en el contexto de la LMC. Giehl y colaboradores mostraron en células primarias de LMC que las alteraciones centrosómicas aumentaban según progresaban las fases de la enfermedad, y que

éstas precedían a las alteraciones cromosómicas (Giehl et al., 2005). Los autores sugirieron que la adquisición de los defectos en los centrosomas contribuye a la inestabilidad cromosómica y puede ser uno de los mecanismos que faciliten la progresión a la fase blástica. El mismo grupo, utilizando células U937 que expresan de forma inducible BCR-ABL1, estableció la relación causa-efecto cuando observaron una reversión de las alteraciones centrosómicas a niveles basales, al retirar el inductor (Giehl et al., 2007). Otro grupo distinto, demostró la existencia de centrosomas supernumerarios y mitosis aberrantes en la línea celular 32D transformada con BCR-ABL1, y que el efecto correlacionaba directamente también con la expresión de la oncoproteína (Wolanin et al., 2010). Aunque parece claro que BCR-ABL1 produce alteraciones centrosómicas, el papel de estas alteraciones en la transición hacia la CB-LMC no esta completamente esclarecido.

4. Relevancia de la ruta de la anemia de Fanconi/BRCA en la estabilidad genética.

4.1. La ruta de la anemia de Fanconi/BRCA.

La Anemia de Fanconi (AF) es una enfermedad autosómica recesiva (excepto el grupo de complementación FA-B) que se caracteriza por la existencia de un fallo medular progresivo (Butturini et al., 1994; Kutler et al., 2003; Rosenberg et al., 2008), alteraciones congénitas del desarrollo (Dokal, 2006) y propensión a padecer cánceres, sobre todo, leucemia mieloide aguda (LMA) y carcinomas escamosos de cabeza y cuello (Dokal, 2008; Rosenberg et al., 2008). Sin embargo, no se han detectado hasta la fecha casos de LMC o LLA-B Ph+ entre estos pacientes.

Las células de pacientes con AF muestran una marcada sensibilidad al estrés oxidativo y a agentes entrecruzantes de las cadenas del ADN, tales como Mitomicina C (MMC), Diepoxibutano (DEB) o el cisplatino (Auerbach et al., 1989; Auerbach and Wolman, 1976; Castella et al., 2011). El entrecruzamiento entre ambas cadenas de ADN o ICL (del inglés *Interstrand crosslink*), se produce como consecuencia de un enlace covalente entre dos bases de las hebras opuestas del ADN, bloqueando los procesos de replicación y transcripción. Sasaki y colaboradores, demostraron que después del tratamiento con MMC, las células de AF presentaban un nivel muy elevado de alteraciones cromosómicas, que incluían roturas cromatídicas, cromosómicas y aparición de cromosomas radiales (Sasaki and Tonomura, 1973). Esta característica no sólo permitió el desarrollo del test de roturas cromosómicas que, hoy en día, constituye un estándar en el diagnóstico de los pacientes, sino que

además, sugería que la reparación defectuosa de las ICLs era la base del fenotipo clínico y celular que se asocia a la AF. En la actualidad, se considera que las proteínas de la AF tienen un papel clave en el mantenimiento de la integridad del genoma en respuesta a estrés replicativo y a entrecruzamientos en el ADN. En este sentido, se han identificado recientemente a los aldehídos, como candidatos endógenos potenciales a causar daño en el ADN que, para ser reparado, requiere la actuación de las proteínas de la AF (Langevin et al., 2011; Ridpath et al., 2007).

Hasta la fecha, se han descrito en los pacientes de AF mutaciones bialélicas en 16 genes: *FANCA, FANCB, FANCC, FANCD1, FANCD2, FANCE, FANCF, FANCG, FANCI, FANCJ, FANCL, FANCM, FANCN, FANCO, FANCP y FANCQ* (Bogliolo et al., 2013; Cybulski and Howlett, 2011; Kee and D'Andrea, 2010). Todos ellos están situados en cromosomas autosómicos excepto *FANCB*, que se localiza en el cromosoma X (Meetei et al., 2004a). Las mutaciones en *FANCA*, *FANCG* y *FANCC* afectan a la mayor parte de los pacientes (Kennedy and D'Andrea, 2005). Los productos de estos genes actúan en una ruta bioquímica común, la ruta de la AF (Figura 15), de tal manera que los pacientes con mutaciones en cualquiera de estos genes presentan fenotipos clínicos y celulares similares, característicos de la enfermedad.

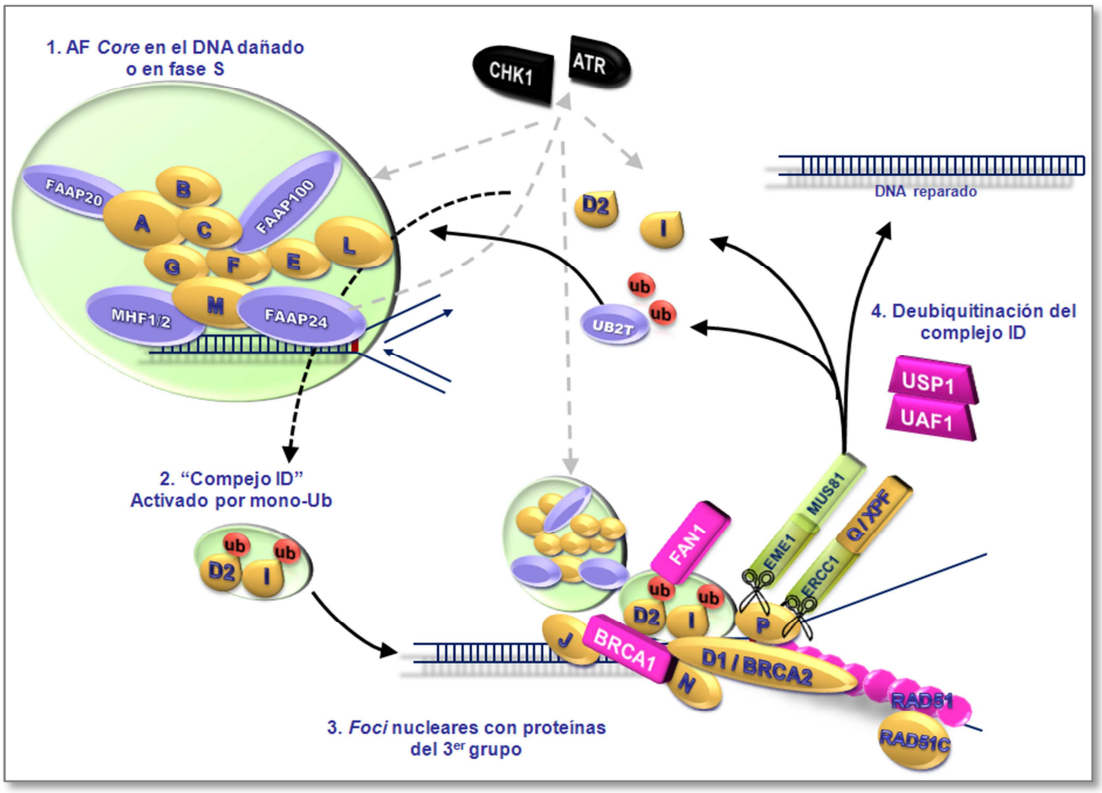

Figura 15: La ruta de la AF/BRCA. Modificado de Valeri y col. (Valeri et al., 2011).

Las proteínas de la AF se pueden clasificar en tres grandes grupos que componen la ruta. El primer grupo comprende las proteínas FANCA, FANCB, FANCC, FANCE, FANCF, FANCG, FANCL, FANCM y tres proteínas asociadas FAAP100, FAAP24 y FAAP20 (Wang, 2007; Yan et al., 2012). Todas ellas forman un complejo multiproteico nuclear que se denomina *core* que se caracteriza por tener actividad ubiquitin-ligasa E3, que reside en FANCL (Meetei et al., 2003). Aparte de FANCL, sólo otra proteína del *core* tiene actividad conocida, FANCM, que es una traslocasa dependiente de ATP (Meetei et al., 2005). El segundo grupo lo componen el heterodímero formado por las proteínas centrales de la ruta, FANCD2 y FANCI que se denomina complejo ID (Dorsman et al., 2007; Garcia-Higuera et al., 2001; Sims et al., 2007; Smogorzewska et al., 2007). El tercer grupo incluye las proteínas FANCD1/BRCA2, FANCN/PALB2, FANCJ/BRIP1, FANCO/RAD51C, FANCP/SLX4 y FANCQ/XPF. Las alteraciones en cualquiera de las proteínas de este tercer grupo no afectan a la monoubiquitinación del complejo ID. Los pacientes de AF que presentan mutaciones en FANCD1/BRCA2 o FANCN/PALB2 se caracterizan por desarrollar leucemias y tumores sólidos en edad temprana del desarrollo (Neveling et al., 2009). Hasta la fecha, todos los genes de este tercer grupo menos *FANCP* (Landwehr et al., 2011) y *FANCQ* (Kohlhase et al., 2014) son supresores de tumores, y sus mutaciones confieren susceptibilidad aumentada a padecer cáncer (Garcia and Benitez, 2008; Meindl et al., 2010; Rahman et al., 2007; Seal et al., 2006). De hecho, se han encontrado asociaciones entre la susceptibilidad a padecer cáncer de mama y ovario hereditario en individuos heterocigotos con mutaciones en FANCD1/BRCA2 y FANCO/RAD51C (uno de los 5 parálogos de RAD51). Ambos funcionan como cofactores de la recombinasa RAD51, clave en la HR (Vaz et al., 2010; Yu et al., 2003). FANCN/PALB2 puede interaccionar con FANCD1/BRCA2, regulando su localización en la cromatina (Xia et al., 2006), y con BRCA1 (Zhang et al., 2009). FANCJ/BRIP1 es una helicasa que interacciona con BRCA1 (Cantor et al., 2004), que como se ha descrito, es una proteína asociada a padecer cáncer de mama y participa también en la HR. FANCP/SLX4 es una endonucleasa con actividad resolvasa durante la HR, que puede actuar como estructura de anclaje de otras endonucleasas (Kim et al., 2011; Stoepker et al., 2011) como XPF-ERCC1, SLX1 o MUS81-EME1. El último miembro descubierto de la ruta es FANCQ/XPF/ERCC4, una conocida nucleasa de la vía de reparación NER, pero que se ha demostrado que participa también en la reparación de la ICL y que las mutaciones en sus distintos dominios confieren hasta tres manifiestaciones clínicas distintas (Bogliolo et al., 2013; Kashiyama et al., 2013).

Como consecuencia de la entrada en la Fase S del ciclo celular o la producción de ICLs, la horquilla de replicación puede quedar bloqueada. Para evitar el colapso replicativo y en respuesta al bloqueo se activa el complejo del *core,* así como la cinasa ATR (Figura 16).

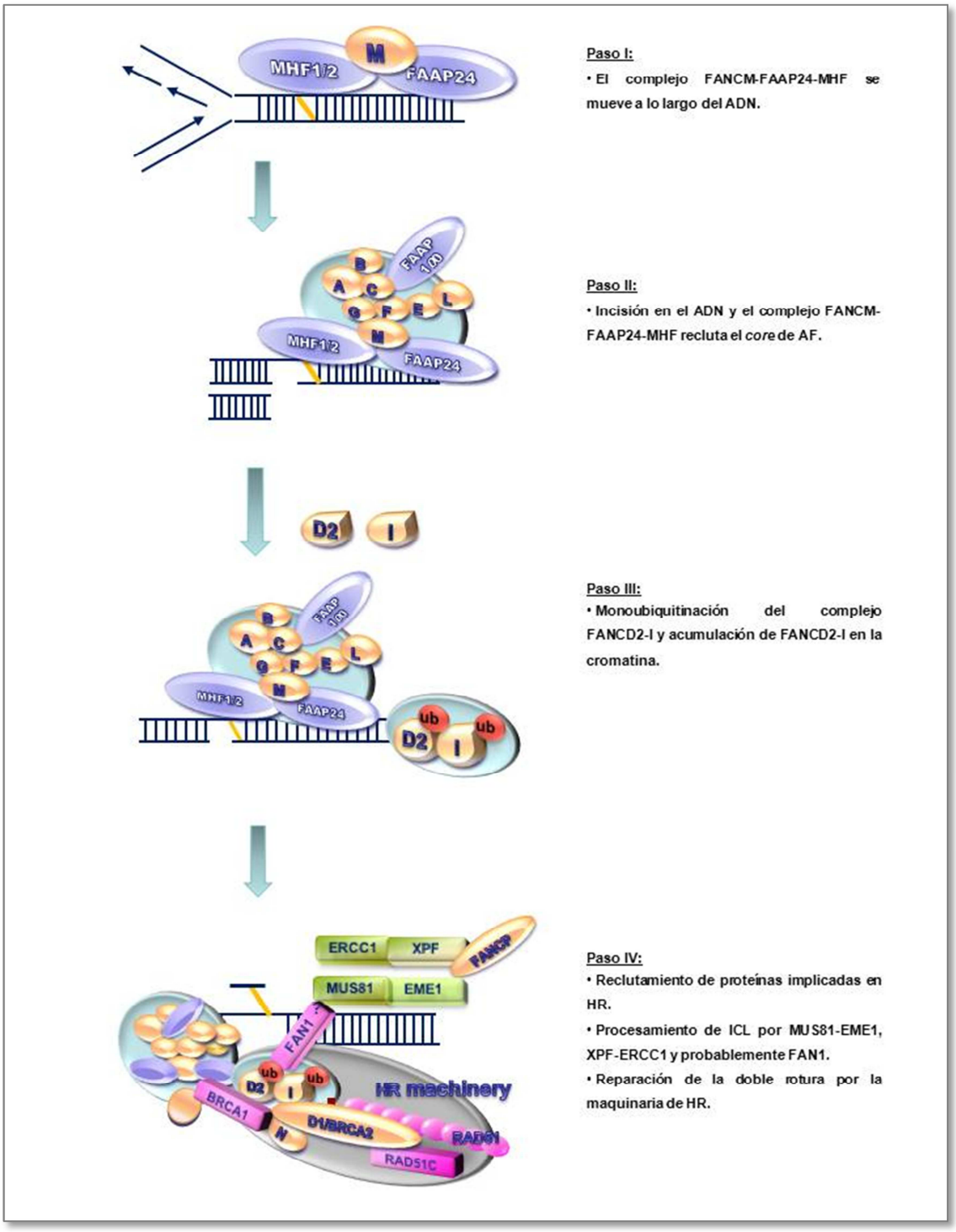

Figura 16: Descripción de los procesos clave de la ruta de AF implicados en la reparación de una ICL (Rio et al., 2011).

ATR es el principal activador de la ruta fosforilando proteínas de la AF como FANCA, FANCE, FANCI, FANCD2 y su principal cinasa efectora CHK1, reponsable del control de ciclo en la fase S. La primera proteína que inicia el proceso de reparación es FANCM que, debido a su actividad traslocasa (Meetei et al., 2005) y junto con FAAP24 y MHF1/2 (Kee and D'Andrea, 2010) se mueve a lo largo de la doble hebra del ADN para detectar horquillas de replicación bloqueadas (Ciccia et al., 2007). El reconocimiento facilita la traslocación y el anclaje del resto del *core* a través de la interacción FANCM-FANCF. La proteína FAAP20 a través de su región N-terminal interactúa con FANCA y contiene un dominio UBZ4, que regula la localización de la polimerasa REV1 en el sitio de daño en el ADN (Kim et al., 2012; Yan et al., 2012). Gracias a la actividad ubiquitin-ligasa del *core* (Meetei et al., 2004b), FANCD2 y FANCI pueden ser monoubiquitinados y posteriormente traslocados a la cromatina, donde forman *foci* nucleares en los sitios de daño (Dorsman et al., 2007; Sims et al., 2007; Smogorzewska et al., 2007). El complejo ID activado induce la pausa en la horquilla de replicación y permite el reclutamiento de la endonucleasa FAN1, que posee regiones de reconocimiento de la ubiquitina de FANCD2, y de otras endonucleasas como MUS81-EME1 y XPF-ERCC1, importantes para el procesamiento de la ICL (Kratz et al., 2010; O'Donnell and Durocher, 2010; Smogorzewska et al., 2010). Estas endonucleasas son las encargadas de la incisión en una de las hebras a ambos lados de la lesión para liberar el aducto y generar una DSB en la otra hebra (Kee and D'Andrea, 2010). En este paso es importante también el reclutamiento de FANCP/SLX4 que puede actuar como anclaje para otras nucleasas y que regula la actividad de XPF-ERCC1 (Cybulski and Howlett, 2011; Kim et al., 2011; Stoepker et al., 2011). El complejo ID activado recluta las polimerasas de síntesis a través de la lesión (TLS), por ejemplo REV1, permitiendo la replicación de la hebra en presencia del monoaducto, que finalmente es eliminado por enzimas de la vía de reparación NER (Kee and D'Andrea, 2010). Los componentes del complejo ID colocalizan con las proteínas del tercer grupo de la ruta y con otras proteínas, como BRCA1 y RAD51, todas ellas asociadas con la HR, que es el siguiente mecanismo por el que se repara la DSB que se genera en el proceso de eliminación de la ICL (D'Andrea, 2010). Finalmente, la ruta se inactiva mediante el complejo enzimático USP1/UAF1 que induce la deubiquitinación de FANCD2 y FANCI (Cohn and D'Andrea, 2008; Nijman et al., 2005; Smogorzewska et al., 2007).

4.2. Papel de la ruta de la AF/BRCA en el control de los diferentes mecanismos de reparación.

La ruta de la AF está implicada en la coordinación de distintos mecanismos de reparación para resolver las ICL, como son la HR, NER y TLS. Estudio previos habían descrito la interacción de FANCJ con distintas proteínas del complejo MutLα de MMR, en concreto MLH1 y PMS2 (Peng et al., 2007), aunque la implicación de las proteínas de MMR en la ruta de reparación de ICLs no está del todo esclarecida. Un estudio reciente además, describe la interacción física y funcional de FANCD2 y FANCI con los complejos MSH2 y MLH1. Los autores sugieren un posible papel de las proteínas del MMR en la activación de la ruta de la AF y en la reparación de las ICLs, y describen por primera vez el defecto del MMR en líneas celulares de AF (Willliams et al., 2011). FANCD2, además, afecta a todos los mecanismos de reparación de las DSBs cuando se ha producido un bloqueo en la horquilla de replicación. Está bien establecido que FANCD2-Ub tiene un papel en la reparación del ADN promoviendo HR y SSA (Nakanishi et al., 2005; Smogorzewska et al., 2007; Yamamoto et al., 2005a; Zhang et al., 2007a). Células deficientes en genes de la AF son sólo ligeramente hipersensibles a radiación ionizante o la Hidroxiurea, puesto que la ruta de la AF sólo es esencial en la regulación de la actividad de la HR si el daño en el ADN se ha producido por la aparición de entrecruzamientos. De hecho, se ha publicado recientemente que estas células muestran un profundo defecto en la HR cuando se induce a través de una ICL (Nakanishi et al., 2011). Por el contrario, FANCD2-Ub parece inhibir el proceso de NHEJ dependiente de DNA-PKcs (Adamo et al., 2010; Pace et al.). Las células humanas deficientes en proteínas de NHEJ no son sensibles a agentes entrecruzantes, confirmando los estudios en levaduras y ratones que indican que el mecanismo de NHEJ no sería necesario para la reparación de las ICLs (Collins, 1993; Frankenberg-Schwager et al., 2005). Sin embargo, algunos trabajos indican que la inhibición del mecanismo de NHEJ en líneas celulares derivadas de pacientes con AF puede reducir la toxicidad de moléculas inductoras de ICLs. Estos estudios proponen que durante la reparación de la ICL, las proteínas de AF previenen la unión inapropiada de los efectores de NHEJ a los extremos del ADN y suprimen la ligación defectuosa de las DSBs inducidas por ICLs, entre cromosomas no homólogos (Adamo et al., 2010; Pace et al., 2010). La posibilidad de utilizar inhibidores de NHEJ para disminuir la severidad del fenotipo de la AF es necesario estudiarla con cautela, pues su uso podría tener consecuencias indeseables en los pacientes. Por ejemplo, se conoce que ratones dobles mutantes en FANCD2 y DNA-PKcs son mucho más sensibles a la radiación ionizante que los mutantes sencillos en DNA-PKcs (Houghtaling et al., 2005). Más aun, un estudio reciente indica que la eliminación de

53BP1 o Ku80 en células deficientes en FANCD2 incrementa de forma marcada su inestabilidad genética, poniendo de manifiesto que la actividad esencial de FANCD2 durante la reparación no puede ser compensada inhibiendo el proceso de NHEJ (Bunting et al., 2012). Recientemente, se ha descrito otro papel importante de FANCD2 en el procesamiento de las DSBs, promoviendo HR. FANCD2-Ub se encargaría de llevar a la proteína CtIP a la cromatina para inciar la resección de la DSB durante el proceso de reparación de la ICL. Las células mutantes deficientes en los dominios de CtIP que se unen a FANCD2, aumentaron los niveles de actividad NHEJ y resultaron hipersensibles a ICLs. Por tanto, no sólo BRCA1 (Bunting et al., 2010; Escribano-Diaz et al., 2013) sino que también FANCD2 participa en la competición NHEJ/HR que se establece entre rutas de reparación durante el procesamiento de la DSB (Murina et al., 2014; Unno et al., 2014).

4.3 Función de la ruta de la AF/BRCA en la regulación del daño oxidativo.

La aparición de estrés oxidativo se produce por una alteración en el balance que supone la producción de ROS y el funcionamiento de los sistemas antioxidantes en la célula. Existe suficiente evidencia científica acumulada que demuestra la hipersensibilidad de las células de AF a agentes oxidantes, el estado pro-oxidativo de las células de la AF, la relación de los síntomas clínicos de esta enfermedad con el daño oxidativo y la interacción de varias de las proteínas de la ruta de la AF/BRCA con proteínas que participan en componentes del proceso de oxidación-reducción o con factores de transcripción que regulan la expresión de enzimas antioxidantes.

El estudio pionero llevado a cabo por Joenje y col. en 1981 (Joenje et al., 1981) puso de manifiesto por primera vez la hipersensibilidad de las células de AF a ROS *in vitro*. En este trabajo, la fragilidad cromosómica de linfocitos T variaba en función de la tensión de oxígeno en cultivo. Varios estudios adicionales han probado la excesiva sensibilidad tanto en cultivos de linfocitos primarios y líneas celulares linfoblásticas de AF a elevadas concentraciones de oxígeno y hierro *in vitro*, ambos agentes oxidantes bien caracterizados. En estos trabajos, la inestabilidad cromosómica y la apoptosis en estas células podía ser corregida adicionando agentes antioxidantes al medio de cultivo como la N-Acetíl cisteína (NAC), la selenometionina o el acido α-lipoico, o disminuyendo la concentración de oxígeno en el ambiente (Clarke et al., 1997; Du et al., 2012; Nordenson, 1977; Ponte et al., 2012; Poot et al., 1996; Saadatzadeh et al., 2004; Schindler and Hoehn, 1988; Takeuchi and Morimoto, 1993). En este sentido, también se conoce que agentes inductores de óxido nítrico (NO) son capaces de inhibir el crecimiento *in vitro* de las células de ratón *Fancc*$^{-/-}$ y que estas especies

reactivas pueden ser inducidas por citocinas como el INF-γ (Hadjur and Jirik, 2003). Dos artículos muy recientes han tratado de caracterizar el origen de la elevada cantidad de ROS en las células de AF. Se ha demostrado que en las células de AF (AF-A, C, y D2) existe un alto nivel de ROS mitocondrial que se asocia con alteraciones en complejos de la cadena respiratoria, disminución en el potencial de membrana mitocondrial, en la producción de ATP e incluso en cambios en la ultraestructura del orgánulo, que vienen acompañados de la inactivación de enzimas esenciales para la producción de energía y para la detoxificación de ROS, como la SOD1 (Kumari et al., 2014; Ravera et al., 2013).

Otros modelos experimentales también han mostrado asociación entre el estrés oxidativo y la ruta de la AF. Por ejemplo, en estudios en células *ex vivo* o fluidos corporales se han encontrado evidencias de un estado pro-oxidado, como el aumento de 8-oxodG, del ratio de glutatión GSSG:GSH, de los niveles de Metilglioxal (Piruvaldehído) o reducción de los niveles de actividad de la CATALASA o de la SOD en plasma, orina o distintos componentes celulares de los pacientes de AF (Degan et al., 1995; Pagano et al., 2004; Petrovic et al., 2011). Asimismo, aunque la mayoría de los modelos en ratón de la AF no reproducen las manifestaciones clínicas hematológicas de la enfermedad, estudios llevados a cabo en ratones deficientes para genes de esta ruta han puesto de manifiesto la interacción entre el defecto en los genes de la AF y el mantenimiento de la hematopoyesis durante el metabolismo oxidativo, así como la aparición de otros signos patológicos del estrés oxidativo *in vivo*. Hadjur y col. (Hadjur et al., 2001) detectaron fallo hematopoyético y esteatosis hepática en ratones *Fancc*$^{-/-}$ *Sod1*$^{-/-}$; Sejas y col. (Sejas et al., 2007) fueron capaces de inducir supresión hematopoyética mediante la producción de ROS inflamatorio en ratones *Fancc*$^{-/-}$. Rani y col. (Rani et al., 2008) demostraron la existencia de respuesta al daño en el ADN y parada de ciclo celular dependiente de p53, en respuesta a daño oxidativo y estrés oncogénico en el modelo de ratón *Fanca*$^{-/-}$ *Trp53*$^{-/-}$. En este mismo modelo, Zhang y col. (Zhang et al., 2008) consiguieron retrasar la aparición de tumores sólidos mediante el efecto protector de antioxidantes como el nitróxido Tempol, o la corrección parcial de los defectos en células madre hematopoyéticas en el modelo *Fancd2*$^{-/-}$, mediante el uso del polifenol antioxidante, Resveratrol (Zhang et al., 2010b).

Se han reportado importantes interacciones moleculares entre componentes de la ruta de AF/BRCA y otras proteínas implicadas en actividades redox y/o moduladores que las regulan. En lo que respecta a componentes del core (FANCA, FANCC, FANCG), dichas interacciones moleculares ocurren sobre componentes citoplásmicos y

mitocondriales. Por ejemplo, la proteína FANCC es capaz de interaccionar con la NADPH citocromo P450 reductasa (RED), con la glutatión S-reductasa (GSTP1) (Cumming et al., 2001; Kruyt et al., 1998) y con la cinasa 1 reguladora de la señal apoptótica (ASK1) (Saadatzadeh et al., 2004). También se ha demostrado que FANCG interacciona funcionalmente con el citocromo P450 2E1 (CYP2E1) (Futaki et al., 2002) y FANCG, FANCA y FANCC se asocian con el antioxidante mitocondrial PEROXIREDOXINA 3 (Mukhopadhyay et al., 2006). Las interacciones entre la proteína central de la ruta, FANCD2 y el estrés oxidativo se han puesto de manifiesto en dos estudios independientes, donde encontraron que ATM (Castillo et al., 2011) y FOXO3a (Li et al., 2010a) ejercen funciones coordinadas con FANCD2 para el funcionamiento del control de ciclo celular en fase S y para la transcripción de enzimas antioxidantes como la SOD1, SOD2, CATALASA o la Glutation-peroxidasa 1 (GPX1) en respuesta a H_2O_2. De hecho, ATM tiene una función esencial para el auto-mantenimiento de las CMHs a través del control de los niveles de ROS (Ito et al., 2004) y FOXO3a es esencial para la regulación de ATM y su control de ROS en CMHs (Yalcin et al., 2008). Por otra parte, FOXO3a es el factor más importante en el control transcripcional de genes antioxidantes y su interacción con FANCD2 en el núcleo induce resistencia celular al daño inducido con H_2O_2 (Li et al., 2010a). En otro trabajo posterior del mismo grupo se demuestra que FANCD2 co-localizando con FOXO3a y el factor remodelizador de la cromatina BRG1 forma un complejo que es capaz de contrarrestar el efecto del estrés oxidativo mediante la protección selectiva de los promotores de los genes antioxidantes pero sin alterar las cinéticas de reparación del daño oxidativo (Du et al., 2012). Esta es una de las cuestiones más polémicas dentro de la hipótesis que postula que los procesos redox y concretamente, la deficiencia en la reparación del daño inducido por ROS, puede ser responsable de muchas de las anomalías clínicas y celulares observadas en la AF. De forma general, se acepta que la principal vía de reparación de 8-oxodG, la vía BER, funciona correctamente en células deficientes en proteínas de AF (Castillo et al., 2011). Recientemente, Mace-Aime (Mace-Aime et al., 2010) y col. han demostrado alteraciones en la glicosilasa de la ruta BER NEIL1, en extractos de linfocitos deficientes en FANCA, C y D2 y, aunque no detectaron diferencias en cuanto a la actividad BER comparado con sus controles sanos, sí su posible participación en la reparación de ICLs. Estos resultados sugieren que el desequilibrio oxidativo en las células de AF se produce porque las proteínas de la ruta pueden estar influyendo en la expresión de enzimas antioxidantes o en la biosíntesis metabólica de ROS y no tanto en la reparación del daño asociado a dicho estrés. La proteína FANCJ/BACH1/BRIP1 incluye múltiples funciones que tienen relación con el estrés oxidativo. Presenta interacción física y funcional en la supresión

de cáncer de mama y ovario, y en la reparación del ADN junto con BRCA1. BRCA1 a su vez, se ha demostrado que interacciona con el factor de transcripción NRF2 promoviendo la señal antioxidante (Gorrini et al., 2013). FANCJ compite directamente con NFR2 produciendo la regulación negativa en los elementos de respuesta antioxidante (ARE) de promotores de genes antioxidantes, como el de la NADPH:quinona-reductasa (Dhakshinamoorthy et al., 2005). Es más, actúa como represor inducible por hipoxia del gen de la hemo-oxigenasa-1 (HO-1) (Kitamuro et al., 2003) y se le ha asignado un papel como inhibidor de la senescencia inducida por estrés oxidativo bloqueando la acción de P53 (Dohi et al., 2008). Por último, FANCN/PALB2 interacciona directamente con KEAP1, que es un sensor de estrés oxidativo que une y reprime a su vez al factor de transcripción de genes antioxidantes NRF2. FANCN compite con NRF2 por la unión de KEAP1, de esta manera permite la acumulación y función de NRF2 en el núcleo disminuyendo los niveles de ROS (Ma et al., 2012). En conjunto, se ha reportado asociación con el estrés oxidativo para seis de las proteínas de la ruta de AF (FANCA, FANCC, FANCG, FANCD2, FANCJ y FANCN) pertenecientes a los tres complejos y resulta evidente que, a medida que se vayan estudiando en profundidad las distintas proteínas de la ruta, se van a ir asignando nuevas funciones más allá de la reparación del daño en el ADN producido por ICLs.

4.4. Alteraciones de la ruta de la Anemia de Fanconi en los cánceres esporádicos.

Los principales mecanismos por los que se altera la ruta de reparación de la AF se han resumido en la tabla 1 y son los siguientes: Silenciamiento epigenético de los genes de la AF, mutaciones somáticas que inducen la pérdida de función de los genes de la AF e incremento en la expresión de variantes del procesamiento alternativo del ARN que promueven la disminución de la expresión de las variantes normales y compiten con las proteínas funcionales de la AF.

Tabla 1: Cánceres esporádicos con alteraciones en la ruta de AF.

Anomalía en célula tumoral	Gen	Tipo de cáncer	Frequencia (%)	Referencia
Hipermetilación del promotor	*BRCA2*	Mama	9/18 (50)	(Cucer et al., 2008)
"	"	*Granulosa*	1/25 (4)	(Dhillon et al., 2004)
"	"	*CECC*	13/13 (100)	(Szaumkessel et al., 2011)
"	"	CECC	4/63 (6,4)	(Szaumkessel et al., 2011)
"	*FANCB*	CECC	1/16 (6,2)	(Smith et al., 2010)
"	*FANCC*	LMA	1/143 (0,7)	(Hess et al., 2008)
"	"	LLA	3/97 (3,1)	(Hess et al., 2008)
"	*FANCF*	*LMA*	1	(Tischkowitz et al., 2003)
"	"	Vejiga	1/41 (2,4)	(Neveling et al., 2007)
"	"	*Vejiga*	1/23 (4,3)	(Neveling et al., 2007)
"	"	Mama	13/75 (17,3)	(Olopade and Wei, 2003)
"	"	Mama	1/120 (0,8)	(Wei et al., 2008)
"	"	Mama	4/99 (4)	(Tokunaga et al., 2011)
"	"	Cérvix	27/91(29,7)	(Narayan et al., 2004)
"	"	*Cérvix*	3/9 (33,3)	(Narayan et al., 2004)
"	"	Células germinales (no seminoma)	4/60 (6,7)	(Koul et al., 2004)
"	"	*Granulosa*	6/25 (24)	(Dhillon et al., 2004)
"	"	CCE	13/89(14,6)	(Marsit et al., 2004)
"	"	CPNM	22/158 (13,9)	(Marsit et al., 2004)
"	"	Ovario	4/19 (21)	(Lim et al., 2008)
"	"	*Ovario*	5/18 (27,7)	(Taniguchi et al., 2003)
"	"	Ovario*	7/53 (13,2)	(Lim et al., 2008)
"	"	*Ovario*	1/7 (14,3)	(Wang et al., 2006)
"	"	*Ovario*	2/25 (8)	(Taniguchi et al., 2003)
"	"	*Ovario*	1/9 (11,1)	(Lim et al., 2008)
"	*FANCL*	LLA	1/97 (1,0)	(Hess et al., 2008)

"	*FANCN*	Mama	4/60 (6,6)	(Potapova et al., 2008)
"	"	Ovario	4/53 (7,5)	(Potapova et al., 2008)
Mutación somática	*BRCA2*	Mama	1/69 (1,4)	(Weber et al., 1996)
"	"	Mama	1/23 (4,3)	(Kwiatkowska et al., 2002)
"	"	Ovario^{+}	4/92 (4,3)	(Hilton et al., 2002)
"	*FANCA*	LMA	4/101 (3,4)	(Tischkowitz et al., 2004)
"	"	LMA	5/79 (6,.3)	(Condie et al., 2002)
"	*FANCC*	*Hepatocelular*$^{\#}$	1/5 (20)	(Palagyi et al., 2010)
"	"	Páncreas*	2/33 (6,1)	(van der Heijden et al., 2003)
"	*FANCD2*	CCE Oral	7/21(33,3)	(Sparano et al., 2006)
"	*FANCG*	CCE Oral	6/21(28,6)	(Sparano et al., 2006)
"	"	Páncreas*	1/33 (3,0)	(van der Heijden et al., 2003)
Ausencia de mRNA sin hipermetilación	*BRCA2*	Ovario	1/18 (5,5)	(Zikan et al., 2007)
"	"	Ovario^{+}	12/92 (13,0)	(Hilton et al., 2002)
Variantes de *splicing*	*FANVL*	Pulmón	13/25 (52)	(Zhang et al., 2010a)
"	"	Prostata	30/45 (66,6)	(Zhang et al., 2010a)
"	"	Osteosarcoma	10/10 (100)	(Zhang et al., 2010a)

Datos extraídos y modificados de Valeri y col.(Valeri et al., 2011).

CECC: Cáncer de células escamosas de cabeza y cuello; LMA: Leucemia mieloide aguda; LLA: Leucemia linfoblástica aguda; LMA: Leucemia mieloide aguda; CCE: Cáncer de células escamosas; CPNM: Cáncer de pulmón no microcítico.

En los estudios marcados en cursiva sólo se utilizaron líneas celulares.
* En 11 casos de líneas de cáncer de páncreas y una selección de 22 tumores de desarrollo temprano se detectó pérdida de heterocigosidad en FANCC y FANCG.
$^{+}$ El ARNm de BRCA2 no se detectó en 12 casos de cáncer esporádico de ovario, uno de los cuales mostró metilación del promotor de BRCA2. En esta cohorte también se incluyen casos de cáncer hereditario.
$^{\#}$ No existen muestras de tejido sano del paciente para determinar el origen de la mutación homocigota.

5. Implicación de los microRNAs en LMC.

5.1. Los miARNs. Biogénesis.

A pesar de que el desarrollo tumoral comienza en la mayor parte de los casos debido a alteraciones somáticas (Croce, 2008), también se ha demostrado que muchos tumores muestran alteraciones en la expresión de diferentes genes supresores debido

a modificaciones epigenéticas, tales como la metilación de islas CpG de sus promotores, que conducen a la pérdida de su función (Jones and Baylin, 2007) o las modificaciones post-traduccionales de las histonas, que generan cambios en la accesibilidad de la cromatina para la regulación de la transcripción de los ARNm (Bonifer and Cockerill, 2011). Durante los últimos años se ha demostrado además, que la alteración en la expresión de los microARNs puede contribuir a la patogénesis de la mayoría de los procesos tumorales (Croce, 2009). El número de microARN registrados sigue aumentando de forma exponencial. Hasta la fecha, se han registrado más de 2000 precursores de miARNs en el genoma humano (Di Leva et al., 2014). Las predicciones bioinformáticas indican que más de la mitad de los productos génicos en el genoma están regulados por microARNs y que cada uno de estos es capaz a su vez de controlar la expresión de cientos de genes (Hansen et al., 2011).

Los microARNs son ARNs de cadena sencilla de pequeño tamaño (19-25 nucleótidos) no codificante. Se generan normalmente mediante la transcripción en la que interviene la ARN polimerasa II (Lee et al., 2004) de un precursor de mayor tamaño (pri-miARN), que es procesado para generar el denominado pre-miARN (de 60-100 nucleótidos de largo) por la nucleasa de ARN Drosha, junto con el co-factor DGCR-8 (Pawlicki and Steitz, 2008). El pre-miARN se transporta al citoplasma a través del complejo Exportina 5-Ran-GTP donde es procesado por la ARNasa tipo III Dicer para generar el miARN maduro (Park et al., 2011). Una vez en el citoplasma, una de las hebras maduras del miARN se incorpora al complejo inductor de silenciamiento del ARN (RISC, del inglés *RNA-induced silencing complex*) junto con la proteína Argonauta (Kim et al., 2009b). RISC reconoce secuencias parcialmente complementarias en la región 3'UTR del ARN mensajero (ARNm) del gen diana produciendo inhibición de la traducción y, en la mayoría de los casos en mamíferos (>84%), también degradación del ARNm (Guo et al., 2010) (Figura 17).

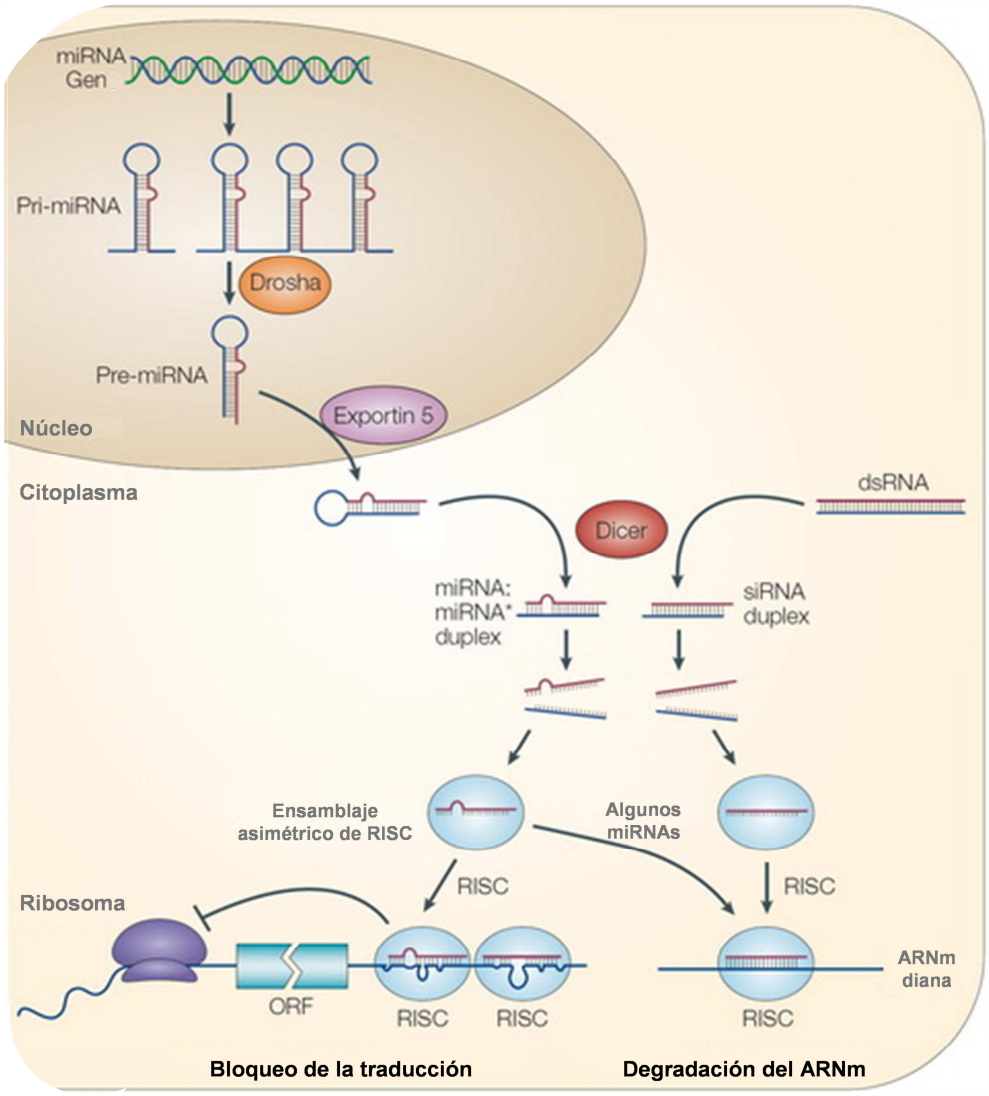

Figura 17. Biogénesis de los miARNs.(He and Hannon, 2004).

5.2. Los miARNs en LMC. Interacción entre BCR-ABL1 y los miARNs.

Los microARNs pueden actuar como oncogenes (oncomiRs) o genes supresores de tumores en el desarrollo de diferentes tipos de neoplasias. A pesar de los múltiples estudios centrados en conocer la implicación de los microARNs en el desarrollo tumoral, el número de trabajos que describen la función de los microARNs en la LMC es limitado y de forma mayoritaria, su expresión está reducida en la patología, tal y como se recoge en la Tabla 2.

Tabla 2. miRNAs con papel funcional en LMC.

mARNs	Localización	Diana/función	Referencia
miR-150 ↓	19q13.33	*MYB*/supresor. MYB induce leucemogénesis	(Machova Polakova et al., 2011)
miR-203 ↓	14q.32.33	*ABL1* y *BCR-ABL1*/supresor.	(Bueno et al., 2008)
miR-328 ↓	16q22.1	*PIM1* y *hnRNP E2*/supresor. Su re-expresión provoca diferenciación	(Eiring et al., 2010)
miR-10a ↓	17q21.32	*USF2*/supresor. USF2 es un factor estimulador de la proliferación	(Agirre et al., 2008)
miR-181a ↓	9q33.3	*RalA* y *MCL1* /supresor. RalA es una GTPasa de la ruta RAS	(Fei et al., 2012; Zimmerman et al., 2010)
miR130a ↑	11q12.1	*CCN3*/oncomiR. CCN3 es un represor de la proliferación	(Suresh et al., 2011)
miR-30a ↓	6q13	*BCR-ABL1*, *ABL1*, *BECN1* y *ATG5*/Supresor. Inhibidor de autofagia	(Liu et al., 2013a; Yu et al., 2012)
miR-138 ↓	3p21.32	*BCR-ABL1*, *ABL1* y *CCND3*/supresor.	(Xu et al., 2014b)
miR-326 ↓	11q13.14	*Smo*/supresor. *Smo* es necesario para el mantenimiento de CML en LMC	(Babashah et al., 2013)
miR-223 ↓	Xq12	*MEF2C* y *PTBP2*/supresor.	(Agatheeswaran et al., 2012)
miR-29a ↓	7q32.3	*RNAase-L* y *BCR-ABL1*/supresor.	(Lee et al., 2013; Li et al., 2013)
miR-196b↓	7p15.2	*HOXA9*, *ABL1* y *BCR-ABL1*/ supresor.	(Liu et al., 2013b)
miR-17-92↑ (grupo)	13q31.3	Se desconoce en LMC/OncomiR. Activado transcripcionalmente por c-MYC	(Venturini et al., 2007)

No obstante, varios estudios han demostrado un perfil típico de expresión de miRNAs en LMC tanto en poblaciones de progenitores hematopoyéticos purificadas como no purificadas de sangre periférica, con un grado de solapamiento parcial de miRNAs entre las distintas investigaciones. Más allá de los diferentes materiales biológicos empleados y las plataformas de *microarrays* utilizadas en cada estudio, el perfil de expresión de los miRNAs publicados distingue claramente entre pacientes con LMC e individuos sanos (Agirre et al., 2008) y más importante todavía, encuentran diferencias entre las distintas fases clínicas de la enfermedad (Machova Polakova et al., 2011) y entre pacientes que responden o no responden a la terapia con inhibidores de tirosina cinasas (Flamant et al., 2010; San Jose-Eneriz et al., 2009), lo que puede resultar

crucial a la hora de explicar la hematopoyesis aberrante en LMC y la aparición de resistencias a los tratamientos.

Venturini y col. demostraron la implicación del grupo policistrónico que comprende 6 miARNs miR-17-92 (también denominado Oncomir-1), como un oncogén importante en la LMC (Venturini et al., 2007). Los ensayos en células $CD34^+$ de pacientes de LMC han confirmado que está incrementada la expresión de miR-17-92 durante la FC-LMC pero no durante la CB-LMC, y su sobre-expresión en la línea K562 promueve la proliferación celular (Venturini et al., 2007). En este estudio se demostró que la transcripción del Pri-miR-17-92 está regulada directamente por c-MYC y también por BCR-ABL1. Además, el tratamiento con imatinib en células mononucleadas de pacientes y líneas celulares de LMC redujo la expresión de algunos de los miembros de este conjunto de miRNAs(Flamant et al., 2010; Venturini et al., 2007). Otro estudio independiente ha reportado que el miR-17-92 está aumentado en leucocitos de sangre periférica de pacientes en CB-LMC, mientras que los niveles eran normales o ligeramente aumentados al diagnóstico (Machova Polakova et al., 2011). El análisis mediante qPCR en células mononucleadas y $CD34^+$ de pacientes con CML ha demostrado la disminución en la expresión de diversos microARNs como miR-10a, miR-150 y miR-151, así como la sobreexpresión del miR-96 (Agirre et al., 2008). Este mismo grupo encontró 19 miARNs diferencialmente expresados entre pacientes que responden y los que son resistentes a la terapia con imatinib (San Jose-Eneriz et al., 2009). De los anteriormente citados, uno de los hallazgos más consistentes entre distintos estudios es la baja expresión de miR-150 en LMC al diagnóstico en comparación con individuos sanos, tanto en MNC de médula ósea o en $CD34^+$ (Agirre et al., 2008), como en leucocitos de sangre periférica total en FA-LMC y CB-LMC (Machova Polakova et al., 2011). Los niveles de miR-150 aumentaban significativamente tras la inhibición de la actividad de BCR-ABL1, tratando la línea Ph^+ MOLM7 con imatinib (Machova Polakova et al., 2011). En este trabajo se demostró que el activador transcripcional MYB es una diana de miR-150. MYB es un factor de transcripción que contribuye a la proliferación y supervivencia de los blastos normales y leucémicos. De hecho, se ha demostrado en modelos de ratón BC-LMC que *c-MYB* es necesario para inducir la leucemogénesis (Lidonnici et al., 2008). Es más, también se ha demostrado una correlación inversa entre los niveles de mir-150 y la expresión de MYB y BCR-ABL1 y una asociación directa entre los niveles transcripcionales de BCR-ABL1 y MYB. Por tanto, la reducción de miR-150 en LMC puede estar contribuyendo al aumento de MYB en LMC (Machova Polakova et al., 2011). Recientemente, Morris y col. (Morris et al., 2013), han publicado que los niveles bajos

o ausentes de expresión de miR-150 contribuyen al bloqueo en la diferenciación mieloide de las células de pacientes en BC-LMC y que este fenotipo está también parcialmente mediado por MYB.

A diferencia de lo que ocurre con el grupo miR-17-92, la disminución en la expresión de miR-10a es independiente de la actividad cinasa de BCR-ABL1 y es proporcional a la expresión de USF2 (*upstream transcription factor 2*), indicando que la disminución en la expresión del miR-10a puede contribuir a la proliferacion celular en la LMC (Agirre et al., 2008). Lopotova y col., por primera vez, sugirieron la existencia de un bucle autoregulador entre la expresión del miR-451 y BCR-ABL1 (Lopotova et al., 2011). En otro estudio con una cohorte pequeña de pacientes encontraron que los niveles de expresión del miR-451 en leucocitos de sangre periférica al diagnóstico se podían asociar a la respuesta a la terapia con imatinib. Además, se demostró que los niveles de miR-451 correlacionaban de forma inversa con los niveles transcripcionales de BCR-ABL1 en pacientes con LMC, aunque la expresión de miR-451 en este trabajo parecía mucho más heterogénea entre los pacientes (Scholl et al., 2012). La actividad cinasa de BCR-ABL1 también es responsable de la disminución de la expresión de miR-31, miR-155 y miR-564 en células de LMC. Esta disminución correlaciona con el aumento en cinco de sus dianas, CBL, E2F3, CCND1, KRAS y AKT2. Estas proteínas son componentes de importantes vías de señalización que incluyen MAPK, ErbB, mTOR y VEGF (Rokah et al., 2012). Debido a su participación en la fisiopatología de la leucemia mieloide aguda (LMA), Fei et al. exploraron la expresión de miR-181a en líneas celulares Ph^+. Los niveles de tránscritos de miR-181a se encontraban suprimidos por debajo del nivel de detección de la RT-PCR. La transfección de miR-181a en la línea K562 disminuyó el crecimiento e indujo apoptosis en las células. En este estudio también se confirmó que RalA, una GTPasa de la ruta de RAS, es una diana directa de miRNA-181a (Fei et al., 2012). La expresión de los miembros de la familia miR-181 también se encontró suprimida en un modelo de resistencia a imatinib en LMC mediada por sobre-expresión de la cinasa Lyn. Concretamente, miR-181b inhibe de forma directa la expresión del componente apoptótico MCL-1, aumentando la supervivencia de las células (Zimmerman et al., 2010). La expresión de miR-130a y miR130b está aumentada por la cinasa BCR-ABL1. El silenciamiento de BCR-ABL1 en líneas Ph^+, disminuye la expresión de miR-130a y aumenta la expresión de su diana, el regulador negativo del crecimiento, CCN3 (Suresh et al., 2011). Recientemente, los miembros de la familia miR-29 han sido identificados como reguladores del oncogén *RNASEL,* en LMC. La expresión ectópica de miRNA-29 en este trabajo, suprimió la expresión de la RNASEL, disminuyó la proliferación en la línea K562 e inhibió el

crecimiento de tumores en un modelo de ratón (Lee et al., 2013).

La detección de la deleción de mal pronóstico en la región del cromosoma 9 (9q 34.1) en una proporción importante de casos de LMC (15-18%), se relacionó con una disminución en la expresión de miR-199b y miR-219-2 (Chaubey et al., 2009). Posteriormente, se determinó que casi la mitad de estos pacientes con deleción de miR-199b eran resistentes al tratamiento con imatinib (Joshi et al., 2014).

El papel del miRNA-328 en la LMC ha sido descrito previamente en el apartado Mecanismos de transición a la fase blástica (Eiring et al., 2010). La ruta de Hedgehog (Hh) es esencial para la supervivencia y auto-renovación de las CMLs de LMC. Babasahah et al. demostraron que la expresión incrementada del transductor de señal, Smoothened (Smo), se asociaba con una reducción en la expresión de miRNA-326 en células $CD34^+$ de pacientes de LMC al diagnóstico. La expresión forzada de miRNA-326 reducía la expresión de SMO, disminuyendo la proliferación y aumentando la tasa de apoptosis en las células $CD34^+$ de LMC. Los resultados demostraron que *SMO* es una diana de miR-326 durante la patogénesis de la LMC (Babashah et al., 2013). En relación a las CMLs de LMC, Zhu et al. demostraron un perfil específico de expresión en 38 pacientes al diagnóstico, en los que tanto la expresión del miR-150, miR-23a y miR-130a como su número de copias absoluto se hallaban reducidos (Zhu et al., 2014).

Se ha identificado otro grupo de miARNs que pueden inhibir directamente la región 3´UTR tanto de BCR-ABL1 como de ABL1. Estos miARNs suelen estar inactivados en los pacientes por pérdida monoalélica e hipermetilación del alelo restante. Ejemplos reportados de estos miR pueden ser: El miR-196b, que tiene como dianas directas además de *BCR-ABL1* y *ABL1*, también *HOXA9*. Las islas CpG del promotor de miR-196b están hipermetiladas en pacientes de LMC (Liu et al., 2013b). El miR-138 que se une al ARNm de *ABL1* en su región codificante y no en la región 3' UTR y también tiene como diana directa a *CCND3*. El miR-138 se induce por el factor de transcripción GATA-1 que a su vez, es reprimido por BCR-ABL1 (Xu et al., 2014a). Otros ejemplos son el miR-29b, que suprime la proliferación de células de líneas de LMC e induce su apoptosis; este miARN se une a la región 3´UTR de *ABL1* y *BCR-ABL1* (Li et al., 2013), o el miR-30a, cuyo nivel de expresión es muy bajo en células de médula ósea de los pacientes y también tiene como diana a *BCR-ABL1* (Liu et al., 2013a). El miR-30a es un conocido inhibidor del proceso autofágico. La autofagia es clave en la resistencia a imatinib en la LMC (Yu et al., 2012). La expresión ectópica de cualquiera de estos miRNA citados podría tener eficacia terapéutica frente a las células de LMC.

Sin embargo, el paradigma de este grupo es el miR-203, silenciado por hipermetilación en LMC, regula la expresión tanto de *ABL1* como de *BCR-ABL1*, por lo que su re-expresión tanto por transfección como con el uso de agentes hipometilantes en células LMC, es capaz de inhibir la proliferación tumoral actuando como un supresor que podría ser utilizado en un futuro para el tratamiento de la enfermedad (Bueno et al., 2008). Imatinib también es capaz de inducir la demetilación del miR-203 (Shibuta et al., 2013).

5.3. Los miARNs como factores teragnósticos en cáncer. Primeros ensayos clínicos.

La evidencia de que diversos microRNAs estén desregulados en LMC y de que la manipulación de los miARNs produce cambios drásticos en los fenotipos tumorales, puede ser explotada para el diseño de terapias de la enfermedad (Garzon et al., 2010; Iorio and Croce, 2012). Existen dos estrategias principales para modular la expresión de los miARNs en cáncer. La primera es una estrategia directa, que conlleva el uso de oligonucleótidos modificados químicamente (LNA-AntimiRs, AntagomiRs, máscaras de miARNs y esponjas de miARNs) y construcciones basadas en virus, para interferir la expresión de un miARN oncogénico o recuperar la pérdida de expresión de un miARN supresor de tumores (miméticos de miARNs). La segunda es una estrategia indirecta, que consiste en el uso de fármacos para regular la expresión de los miARNs controlando su transcripción o su procesamiento (agentes hipometilantes, ácido trans-retinoico) (Garzon et al., 2010). Estudios desarrollados en modelos de ratón indican que los miRNAs y los anti-miRNAs muestran modestos efectos adversos y pueden ser eficaces en terapias antitumorales (Huynh et al., 2010; Ma et al., 2010; Trang et al., 2011). Además, los microRNAs podrían ser utilizados también como biomarcadores tumorales en LMC, puesto que su perfil de expresión está asociado al desarrollo de la leucemia (Agirre et al., 2008), a su progresión (Machova Polakova et al., 2011) y a las respuestas a las terapias (San Jose-Eneriz et al., 2009). Es decir, los miARNs tienen establecidas funciones como biomarcadores diagnóstico, pronóstico y predictivos, como se ha demostrado en distintos tipos de tumores (Dillhoff et al., 2008; Lu et al., 2005; Rosenfeld et al., 2008; Varadhachary et al., 2011), donde se pueden analizar niveles estables de miRNAs circulantes en distintos fluidos corporales de una forma accesible y no invasiva (Brase et al., 2010; Etheridge et al., 2011; Kosaka et al., 2010).

Una de las principales ventajas de los miARNs como agentes terapéuticos radica en su habilidad para controlar múltiples genes, normalmente en el contexto de una misma red de conexiones, por lo que son muy eficientes regulando los distintos procesos

biológicos celulares esenciales en la homeostasis de la célula normal o tumoral, como la diferenciación, la proliferación o la supervivencia (Garzon et al., 2010; Iorio and Croce, 2012). Sin embargo, a pesar de que hay acumulada suficiente evidencia para proponer a los miARNs como agentes terapéuticos, existen todavía una serie de obstáculos que impiden el desarrollo al máximo de su potencial clínico. Deben producirse mejoras en cuanto a las modificaciones químicas de estas moléculas que aumenten su estabilidad y reduzcan su degradación *in vivo*. Estas moléculas son degradadas rápidamente por nucleasas presentes en las células o el suero. También deben desarrollarse diferentes estrategias que aseguren la efectividad y la especificidad de su liberación en los diferentes tejidos, a través de administracion sistémica, en órganos menos accesibles. Se están desarrollando métodos para evitar la activación de la respuesta inmune innata por parte de estas moléculas, fundamentalmente de fagocitos. Además, su tamaño pequeño permite que sean filtradas fácilmente por el riñon y ser excretadas. Más aún, la carga negativa de estas móleculas dificulta su paso a través de membranas, por lo que se reduce su captación en la célula. Por último, se debe llevar a cabo una evaluación de posibles efectos adversos, incluidos aquellos efectos provocados por el control sobre dianas inespecíficas o por los potenciales peligros del uso de partículas virales o liposomas (Garzon et al., 2010; Iorio and Croce, 2012).

A pesar de todos estos posibles inconvenientes, la terapia con miARNs es una realidad hoy en día. De los más de 200 ensayos clínicos registrados con miARNs, sólo un 10% incluyen manipulaciones directas de estas moléculas como aproximación terapéutica. El resto, en su gran mayoría, son ensayos que contemplan a los miARNs como biomarcadores para el diagnóstico, prónostico y respuesta a distintas terapias en muy diversas patologías, incluidas las neoplasias, donde se está cubriendo un amplio abanico de histiotipos (Registro www.*Clinicaltrials.gov*).

El primer ensayo clínico terapéutico con estas moléculas correspondió a Miravirsen (SPC3649, Santaris Pharma). Es un LNA-oligonucleótido modificado para inhibir el miR-122 en hepatitis crónica causada por el virus de la hepatitis C (HCV) (Lanford et al., 2010). El miR-122 es muy abundante en hígado y es esencial para facilitar el ciclo replicativo del virus. Los primeros ensayos en fase I demostraron que la mólecula, administrada subcutáneamente, era tolerada y no produjo efectos adversos obvios (NCT00688012, NCT00979927). Los resultados en fase II han corroborado la seguridad en humanos y muestran signos prometedores de eficacia con una reducción del ARNm del HCV de hasta tres logaritmos en las dosis más altas (NCT01200420)

(Janssen et al., 2013). El primer ensayo clínico en cáncer se está llevando a cabo con MRX34 (Mirna Therapeutics). En este caso, MRX34 es un compuesto mimético del miR-34 en liposoma. El miR-34 es un conocido supresor de tumores que es activado transcripcionalmente por p53 (He et al., 2007). El efecto antitumoral del mimético del miR-34 ya había sido previamente demostrado en modelos pre-clínicos de ratón con tumores de pulmón y próstata (Liu et al., 2005; Trang et al., 2011). Basados en los resultados de MRX34, la compañía está reclutando actualmente participantes para el estudio en fase I, en pacientes con carcinoma hepático primario y carcinoma hepático metastático proveniente de otros tumores, o enfermedades hematólogicas, incluida la LMC en fase acelerada y blástica (NCT01829971). Finalmente, un grupo de nuevos compuestos ha superado los estudios pre-clínicos y se espera que sus programas de investigación alcancen fases clínicas a corto plazo. Por ejemplo, anti-miR-21 (Regulus therapeutics), que ha demostrado eficacia para prevenir la invasión/migración del glioma en ratón (Gabriely et al., 2008). El anti-miR-10b (Regulus therapeutics) suprime las metástasis de pulmón originadas por tumores mamarios (Ma et al., 2010). El anti-miR-380-5p (Regulus therapeutics) que inhibe el neuroblastoma (Swarbrick et al., 2010). Otro ejemplo es del anti-miR-182 (Regulus therapeutics) que suprime las metástasis de pulmón que provienen de melanoma (Huynh et al., 2010), o en el entorno de las enfermedades hematológicas, MGN-4893 (miRagen Therapeutics), que es un LNA-anti-miR-451. Este oligonucleótido bloquea la diferenciación eritroide y se ha propuesto como posible terapia en un síndrome mielodisplásico, la Policitemia vera (Patrick et al., 2010). Todavía no existen registros de ensayos con miARNs específicos para terapia anti-leucémica y sólo en un futuro próximo veremos si estas móleculas en combinación o como agentes únicos, pueden competir con la eficacia de los inhibidores selectivos de proteínas en cáncer.

IV. OBJETIVOS

Teniendo en cuenta el papel de la ruta AF/BRCA en el control de la estabilidad genética de la célula y las observaciones que muestran alteraciones genéticas y epigenéticas en la ruta AF/BRCA en distintos cánceres esporádicos, en esta memoria se han planteado dos objetivos principales:

1. Esclarecer el papel de la ruta AF/BRCA en la inestabilidad genética de células de LMC. Para ello se han planteado tres objetivos parciales:
-Estudiar la expresión y la función de la proteína FANCD2 en progenitores hematopoyéticos portadores de la translocación.
-Investigar la sensibilidad de los progenitores BCR-ABL1 frente a agentes productores de entrecruzamientos en las cadenas de ADN.
-Analizar la implicación de la ruta AF/BRCA sobre la inducción de aberraciones centrosómicas y cromosómicas en progenitores hematopoyéticos portadores de la translocación.

2. Investigar qué nuevas vías de señalización alteran la función de la ruta AF/BRCA en células BCR-ABL1. Para ello se han planteado estos tres objetivos parciales:
-Analizar la implicación potencial de un grupo de miARNs en la regulación de la expresión de proteínas de la propia ruta AF/BRCA o de otras proteínas que las regulen.
-Estudiar la función de nuevos mecanismos de regulación epigenéticos sobre la respuesta al daño en el ADN y la estabilidad genética de células BCR-ABL1.
-Estudiar el papel de los mecanismos epigenéticos sobre la capacidad de proliferación y supervivencia de progenitores hematopoyéticos BCR-ABL1.

V. ARTÍCULO 1

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PLoS one

Bcr/Abl Interferes with the Fanconi Anemia/BRCA Pathway: Implications in the Chromosomal Instability of Chronic Myeloid Leukemia Cells

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Abstract

Chronic myeloid leukemia (CML) is a malignant clonal disorder of the hematopoietic system caused by the expression of the *BCR/ABL* fusion oncogene. Although it is well known that CML cells are genetically unstable, the mechanisms accounting for this genomic instability are still poorly understood. Because the Fanconi anemia (FA) pathway is believed to control several mechanisms of DNA repair, we investigated whether this pathway was disrupted in CML cells. Our data show that CML cells have a defective capacity to generate FANCD2 nuclear foci, either in dividing cells or after DNA damage. Similarly, human cord blood $CD34^+$ cells transduced with *BCR/ABL* retroviral vectors showed impaired FANCD2 foci formation, whereas FANCD2 monoubiquitination in these cells was unaffected. Soon after the transduction of $CD34^+$ cells with *BCR/ABL* retroviral vectors a high proportion of cells with supernumerary centrosomes was observed. Similarly, *BCR/ABL* induced a high proportion of chromosomal abnormalities, while mediated a cell survival advantage after exposure to DNA cross-linking agents. Significantly, both the impaired formation of FANCD2 nuclear foci, and also the predisposition of *BCR/ABL* cells to develop centrosomal and chromosomal aberrations were reverted by the ectopic expression of *BRCA1*. Taken together, our data show for the first time a disruption of the FA/BRCA pathway in *BCR/ABL* cells, suggesting that this defective pathway should play an important role in the genomic instability of CML by the co-occurrence of centrosomal amplification and DNA repair deficiencies.

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Introduction

Chronic Myeloid Leukemia (CML) is a clonal hematopoietic disorder generated by a t(9;22)(q34;q11) translocation resulting in a *BCR/ABL* oncogene[1,2] that encodes for a tyrosine kinase BCR/ABL-p210 oncoprotein[3]. Although several genetic defects are accumulated in CML cells during the progression from the chronic phase towards the accelerated and blast crisis phases (see review in [4]), studies in mice transplanted with *BCR/ABL* transduced cells demonstrated that this oncogene is the causative agent of CML[5].

In addition to a differentiation arrest, failures in the genomic surveillance and DNA repair of CML cells account for the natural malignant progression of the disease (see review in[6]). Although the mechanisms by which BCR/ABL interferes with the genomic stability of the cell are still poorly understood, the effects of this oncoprotein upon DNA damage, apoptosis and DNA repair are considered critical processes facilitating the accumulation of mutations during the progress to blast crisis (see review in [7]). Moreover, increasing evidence has been published showing that BCR/ABL induces reactive oxygen species (ROSs) causing oxidative damage to CML cells[8], and therefore a variety of DNA lesions, including the highly mutagenic double strand breaks (DSBs)[9,10]. These effects, together with the reported effect of this oncoprotein on the efficacy and/or the fidelity of different DNA repair mechanisms[9,11,12] contribute to explain the mutator phenotype of CML cells.

Concerning the mechanisms by which BCR/ABL affects the repair of the DSBs, previous studies have shown that this oncoprotein interferes both with the non-homologous end joining (NHEJ) pathway and with pathways that utilize homologous templates. Regarding the effects of BCR/ABL on classic NHEJ, Deutsch *et al* observed that the catalytic subunit of DNA-

dependent protein kinase (DNA-PKcs), a key protein in this major DNA repair system in mammalians, was down-regulated in CML cells[13]. In addition to NHEJ, BCR/ABL has also been involved in the aberrant regulation of the two pathways that utilize homologous templates, the faithful homology directed repair (HDR) and the mutagenic single strand annealing (SSA). Interestingly, previous studies have shown that BRCA1, a critical protein for preserving the genomic integrity by promoting homologous recombination[14], is nearly undetectable in CML cells[15]. On the other hand, more recent studies have shown that BCR/ABL specifically promotes the repair of DSBs through SSA, a mutagenic pathway that involve sequence repeats[16,17].

Because the Fanconi anemia (FA) pathway is believed to control several DNA repair pathways, and therefore the genomic stability of the cell (see review in[18]), we ought to investigate the integrity of this pathway in CML cells. Thirteen FA proteins have been identified in the FA pathway, each of them participating in one of the three FA protein complexes. The upstream complex – the FA core complex - is integrated by eight FA proteins (FANCA, FANCB, FANCC, FANCE, FANCF, FANCG, FANCL, FANCM) and two FA associated proteins (FAAP24 and FAAP100). A second complex is formed by FANCD2 and FANCI, which work together in the FA-ID complex. Because of the E3 ligase activity (FANCL) of the FA core complex, FANCD2 and FANCI can be monoubiquitinated and then loaded onto chromatin, forming large nuclear foci in response to DNA damage or replication arrest. Finally, monoubiquitinated FANCD2/FANCI interact with downstream FA proteins such as FANCJ/BRIP1, FANCN/PALB2 and FANCD1/BRCA2, which form stable complexes with proteins participating in HDR, like BRCA1 and RAD51[19,20].

The results presented in this study demonstrate for the first time that CML cells are characterized by a defective FA/BRCA pathway, downstream FANCD2 monoubiquitination. In particular we demonstrate that BCR/ABL interferes with the formation of nuclear FANCD2 foci, in a process that can be reverted by the ectopic expression of *BRCA1*. The consequences of this defect upon the genetic stability of *BCR/ABL* cells are shown.

Materials and Methods

Culture of CD34$^+$ cells from CML patients and healthy umbilical cord blood

Studies were approved by the authors' Institutional Review Board and conducted under the Declaration of Helsinki rules. Chronic myeloid leukemia CD34$^+$ cells were obtained from the peripheral blood (PB) of CML patients, after previous informed consents approved by the ethical Committee of the Clínica Universitaria de Navarra. Healthy CD34$^+$ cells were obtained from umbilical cord bloods (CB) scheduled for discard, after written informed consents of the mother. Mononuclear cells (MNCs) were obtained by fractionation in Ficoll-hypaque according to manufacturer's instructions (GE Healthcare, Stockholm, Sweden). Purified CD34$^+$ cells were obtained using a MACS CD34 Micro-Bead kit (Miltenyi Biotec, Gladbach, Germany). For the expansion of CML CD34$^+$ cells, samples were cultured in StemSpan medium (Stem Cell Technologies, Vancouver, BC, Canada) supplemented with 100 ng/ml human stem cell factor (hSCF; Peprotech, London, UK), 100 ng/ml Flt3-L (Invitrogen, Carlsbad, CA), 20 ng/ml IL-6 (Peprotech) and 20 ng/ml IL-3 (Biosource).

Cell lines

Human-derived Mo7e (a megakaryoblastic leukaemia cell line without the *BCR/ABL* fusion) and Mo7e-p210 cells (Mo7e cells transfected with p210 isoform of *BCR-ABL1*) [21,22] were cultured in RPMI medium (Gibco, NY) supplemented with 10% fetal bovine serum (FBS; Lonza, Belgium), 2 mM L-glutamine (Gibco), 100 U/mL penicillin/streptomycin (Gibco) at 37°C in a humidified atmosphere with 5% CO_2. Mo7e cells were grown in medium supplemented with 10 ng/ml of hr-IL3 (Biosource). FA-A LCLs were cultured in RPMI medium (Gibco) supplemented with 15% FBS, 2 mM L-glutamine (Gibco), 100 U/mL penicillin/streptomycin (Gibco) at 37°C in a humidified atmosphere with 5% CO_2.

Retroviral vectors production

The control retroviral vector (RV) used in these experiments was the MIG-R1 retroviral vector which consist on a MSCV-IRES-GFP vector. The MIG-210 is derived from the MIG-R1, and contains the full-length b3a2 *BCR/ABL* cDNA under the control of the MSCV promoter. Both vectors were kindly provided by Bryan G. Druker (Oregon Health and Science University, Portland, OR). Where indicated, cells were transduced with similar RVs (MIN-210 and its respective MIN-R1 control, kindly provided by W. S. Pear). In these vectors, a truncated version of the NGFR (*Δ*NGFR) cDNA was used instead of the EGFP marker gene, to facilitate the immunoselection of transduced cells. In order to ectopically express BRCA1 in control and BCR/ABL positive cells, S11Brca1-IRES-Neo and S11-IRES-Neo RVs were generated. Retroviral vectors were produced and titrated as previously described[23].

Retroviral transduction of hematopoietic progenitors from human cord blood

Human CB CD34$^+$ cells were pre-stimulated for 48 h with StemSpan (StemCell technologies) supplemented with 300 ng/ml hSCF (Peprotech), 100 ng/ml hTPO (R&DSystems, Minneapolis, MN) and 100 ng/ml Flt3-L (Invitrogen, Carlsbad, CA). Pre-stimulated cells were re-suspended at a density of 5×10^5 cells/ml in retroviral supernatant medium supplemented with FBS (20% final concentration) and growth factors. Cells were then added to retronectin-coated wells (Retronectin, Takara Shuzo, Otsu, Japan) preloaded with the correspondent retroviral vector. Supernatants were replaced every 12 h by new virus containing medium. A total of four transduction cycles were routinely conducted[23].

When hCB CD34$^+$ cells were transduced with MIN-R1 and MIN-210 and purified by immunomagnetic cell sorting with anti-NGFR beads (Miltenyi Biotech) 2 days after transduction following manufacturer's instructions. Purified populations contained at least 95% of NGFR$^+$ cells.

DNA damage treatments and drugs exposure

To conduct Western Blotting and Immunofluorescence assays, cells were treated with Imatinib 1 μM (Novartis Pharma, Basel, Switzerland) for 24 hours and then treated with 40 nM of mitomycin C (MMC) for 16 hours. For inhibition studies cells were treated for 16 hours with MMC and afterwards 1 hour with 40 μM of MG132 (Sigma-Aldrich, ST.Louis, MO) or 20 μM of LY294002 (Cell Signaling Technology, Inc, Danvers, MA). To test the cellular resistance to MMC, clonogenic assays were conducted in semisolid medium (Methocult H4434, StemCell Technologies) containing increasing concentrations of the drug, plated in triplicate on 35-mm plastic culture dishes (Nunc, Roskilde, Denmark) and cultured at 37°C, 5% CO_2 and fully humidified air. Fourteen days after plating, the total number of colonies was scored under an inverted microscope.

Chromosomal breakage analyses

CB CD34$^+$ cells were transduced with MIN-210 or its respective MIN-R1 control and purified two days afterwards, prior to re-infect

these samples with *Neo*^{r} or *BRCA1/Neo*^{r} vectors. Samples were left untreated or treated with 0.1 μg/ml of diepoxibutane (DEB) for a 72-h period. To obtain metaphases, colcemid (0.1 μg/ml; Gibco) was added 24 h prior to harvesting. Cells were then treated with hypotonic solution (0.075 M KCl, Sigma), fixed in methanol:acetic acid (3:1 vol/vol), dropped onto clean slides and air-dried O.N. following standard cytogenetic procedures. Slides were stained with 10% Giemsa in phosphate buffer, pH 6.8. Fifty to seventy metaphases per sample were analyzed for chromosome aberrations including gaps, chromosome and chromatid breaks, acentric fragments, and chromosome- and chromatid-type exchanges.

Flow cytometry analyses

For cell cycle analyses, aliquots of 10^5 cells were washed twice in PBS and fixed in 4.5 mL ice-cold 1% methanol-free formaldehyde in PBS for 15 min on ice. After centrifugation, 5 mL of 70% ethanol (0–4°C) was added, and cells were stored at −20°C at least for 2 hours. After washing, cells were resuspended in 1 mL propidium iodide solution (5 μg/mL; Molecular Probes) with 100 μg/mL DNase-free RNase A (Sigma) and incubated for 30 min at 37°C. Finally, cells were analyzed by flow cytometry (Coulter XL) with linear fluorescence of propidium iodide (DNA content) from 10,000 events with doublet discrimination.

Western Blot analyses

Western blot analyses were performed using extracts of purified $CD34^+$ cells collected by centrifugation. After electrophoresis, proteins were transferred to a nitrocellulose membrane. After blocking, the membrane was incubated at 4°C O.N. with the primary antibodies (anti-FANCD2, Abcam ab2187) diluted in blocking solution, washed extensively and incubated with the appropriate secondary antibody using the Western Breeze Immunodetection Kit (Invitrogen), according to methods previously described[24].

Immunofluorescence studies

In these studies cells were fixed with 3.7% paraformaldehyde in PBS for 15 minutes followed by permeabilization with 0.5% Triton X-100 in PBS for 5 min. After blocking for 30 minutes in Blocking buffer (10% FBS, 0.1% NP-40 in PBS) cells were incubated with rabbit polyclonal anti-FANCD2 (Abcam, ab2187-50), mouse monoclonal anti-γH2AX (Upstate, JBW301), and mouse monoclonal anti-BRCA1 (Oncogene, ab-1 and ab-3). For centrosomal staining, cells were fixed and permeabilized and incubated with primary antibody against γ-tubulin (Abcam, ab16504). Cells were subsequently washed three times in TBS (50 mM Tris-HCl (pH 8.0), 150 mM NaCl) and incubated with anti-mouse or anti-rabbit Texas Red (Molecular Probes, Leiden, The Netherlands) as secondary antibodies. After 45 min. cells were washed three times with TBS and the slides were mounted in Moviol with 4,6-diamidino-2 phenylindole (DAPI). Slides were analyzed with a fluorescence microscope Axioplan2 (Carl Zeiss, Göttingen, Germany) using a 100x/1.45 oil working distance 0.17-mm objective. The proportion of cells with nuclear foci was scored as described elsewhere[24] after analyzing 200 cells per slide. Immunofluorescent images were acquired with an AxioCam MRm (Carl Zeiss) and were processed with AxioVision version 4.6.3 (Carl Zeiss) and Corel Photo-Paint 11 (Corel, Ottawa, Canada).

Statistical analysis

Results are shown as the mean±s.e. Differences between groups were assessed using the two tailed Student's t-test. The data from the clonogenic assays were calculated as survival percentages respect to control cultures. The survival data were fitted by least squares only for experiments with at least three available data points. The IC50 value was obtained algebraically, solving the fitted quadratic equation for the value of dose where the estimated percentage of surviving cells would equal 50%. The processing and statistical analysis of the data was performed by using the Statgraphics Plus 5.0 software package (Manugistics, Inc. Rockville, MD).

Results

BCR/ABL interferes with the formation of FANCD2 nuclear foci in hematopoietic progenitors from chronic myeloid leukemia patients

Because of the relevance of the FA/BRCA pathway in the control of DNA repair, we first investigated whether CML cells had a disruption in this pathway. To this aim, and given that the loading of FANCD2 to chromatin constitutes a central process in the FA/BRCA pathway[20], we determined the proportion of cells with nuclear FANCD2 foci, both in a cell line stably transfected with the *BCR/ABL* oncogene (Mo7e-p210 cells) and in the control parental cells (Mo7e cells). As shown in Figure 1a, the proportion of Mo7e-p210 cells with FANCD2 nuclear foci was significantly reduced compared to control Mo7e cells. This effect was evident in samples not exposed to any DNA damaging agent, and also in cells treated with the DNA cross-linking drug MMC.

To investigate whether data obtained in Mo7e-p210 cells was reproduced in primary CML cells, similar studies were conducted with peripheral blood (PB) $CD34^+$ cells from CML patients at diagnosis, and from healthy $CD34^+$ cells obtained from cord blood (CB) samples. Similar to Mo7e cells, the proportion of $CD34^+$ cells with FANCD2 foci was significantly reduced when samples were obtained from CML patients, compared to healthy $CD34^+$ cells. As in Mo7e cells, differences between both groups were significant, both in untreated and in MMC-treated cells (Figure 1b).

The inhibition of FANCD2 foci in Mo7e-p210 cells and in primary CML cells could result from either a direct effect of the *BCR/ABL* oncogene, or through accumulated mutations that may have occurred along the culture of the cell line or during the progression of the disease of CML patients. To understand whether this effect was directly generated by the *BCR/ABL* oncogene, CB $CD34^+$ cells were transduced with a control MIG-R1 vector (only expressing the *EGFP* marker gene) or with the oncogenic MIG-210 vector (expressing both the *BCR/ABL* and the *EGFP* genes), and exposed to MMC seven days afterwards. Additionally, one aliquot of these samples was incubated with imatinib 24 h prior to MMC treatment, to evaluate whether potential effects mediated by BCR/ABL were dependent on the tyrosine kinase activity of the oncoprotein (See schematic experimental protocol in Figure 2a). To ensure that FANCD2 foci were scored exclusively in cells that had been transduced with either MIG-R1 or MIG-210 vectors, only green fluorescent cells were considered for the analysis of nuclear FANCD2 foci.

Consistent with the results in Figure 1, the proportion of MMC-treated $CD34^+$ cells with FANCD2 foci was significantly inhibited when samples were transduced with MIG-210, as compared to the control MIG-R1 vector (Figure 2a). Moreover, while imatinib did not significantly affect the formation of nuclear FANCD2 foci in cells transduced with the control vector, this drug significantly increased the proportion of MIG-210 transduced $CD34^+$ cells with FANCD2 foci (see Figure 2a and representative pictures in Figure 2b). The effect of BCR/ABL upon the formation of nuclear FANCD2 foci was confirmed in MIG-210-transduced $CD34^+$ cells, by the observation that only EGFP-negative (untransduced) cells contained evident nuclear FANCD2 foci; while EGFP

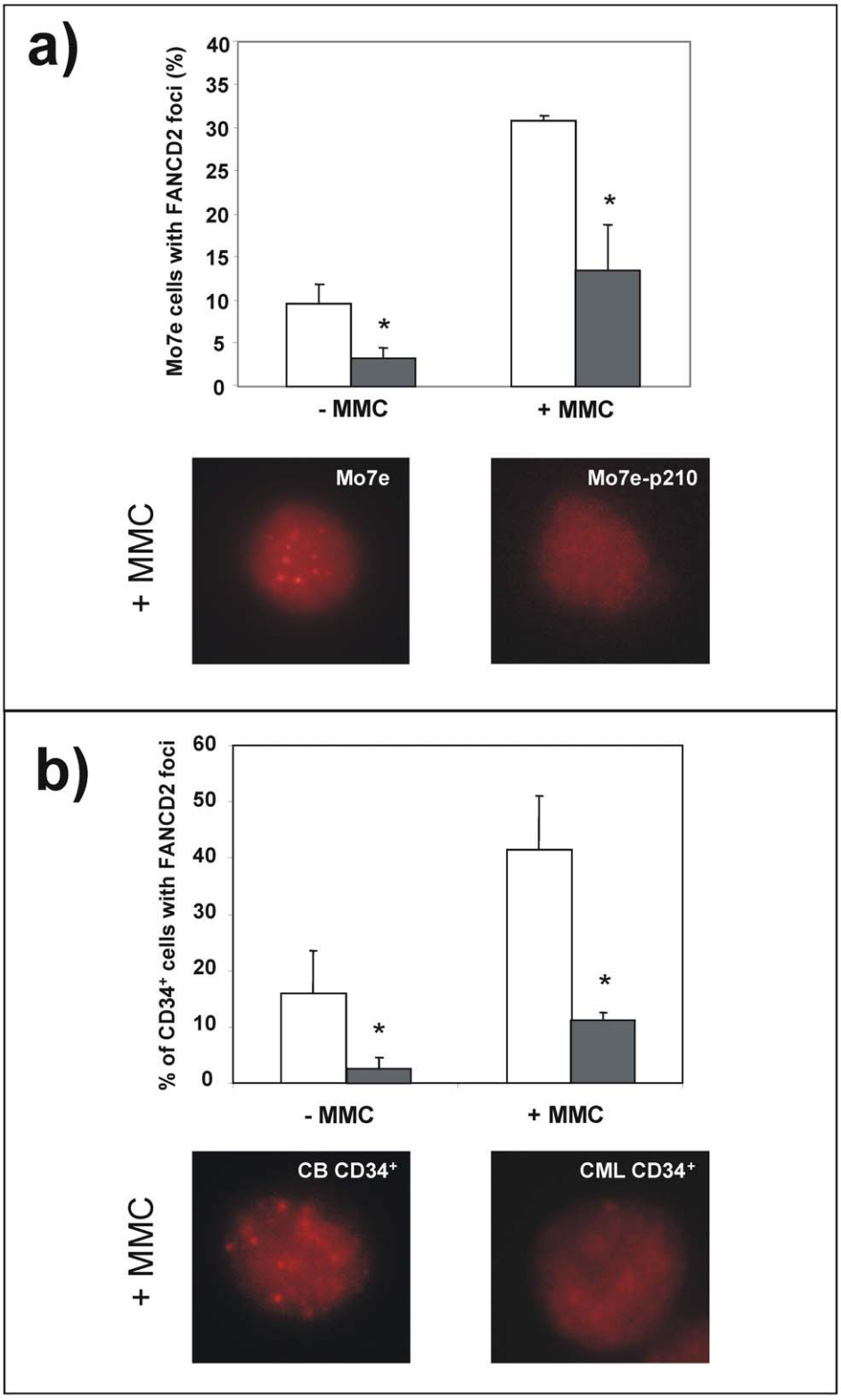

Figure 1. Impaired formation of nuclear FANCD2 foci in Mo7e-p210 and CD34$^+$ cells from CML patients. a) Analysis of the proportion of Mo7e (white bars) and Mo7e-210 cells (grey bars) with FANCD2 nuclear foci, either untreated or treated with MMC (40 nM; 16 h). Panel b) shows the proportion of CD34$^+$ cells from healthy cord blood (white bars) or from the peripheral blood of CML patients (grey bars) with nuclear FANCD2 foci. Bars show mean $\pm$ s.e. of values corresponding to three independent experiments. *Differences between healthy and CML CD34$^+$ cells were significant at $p<0.01$. Representative pictures of nuclear FANCD2 foci in MMC-treated samples are shown.
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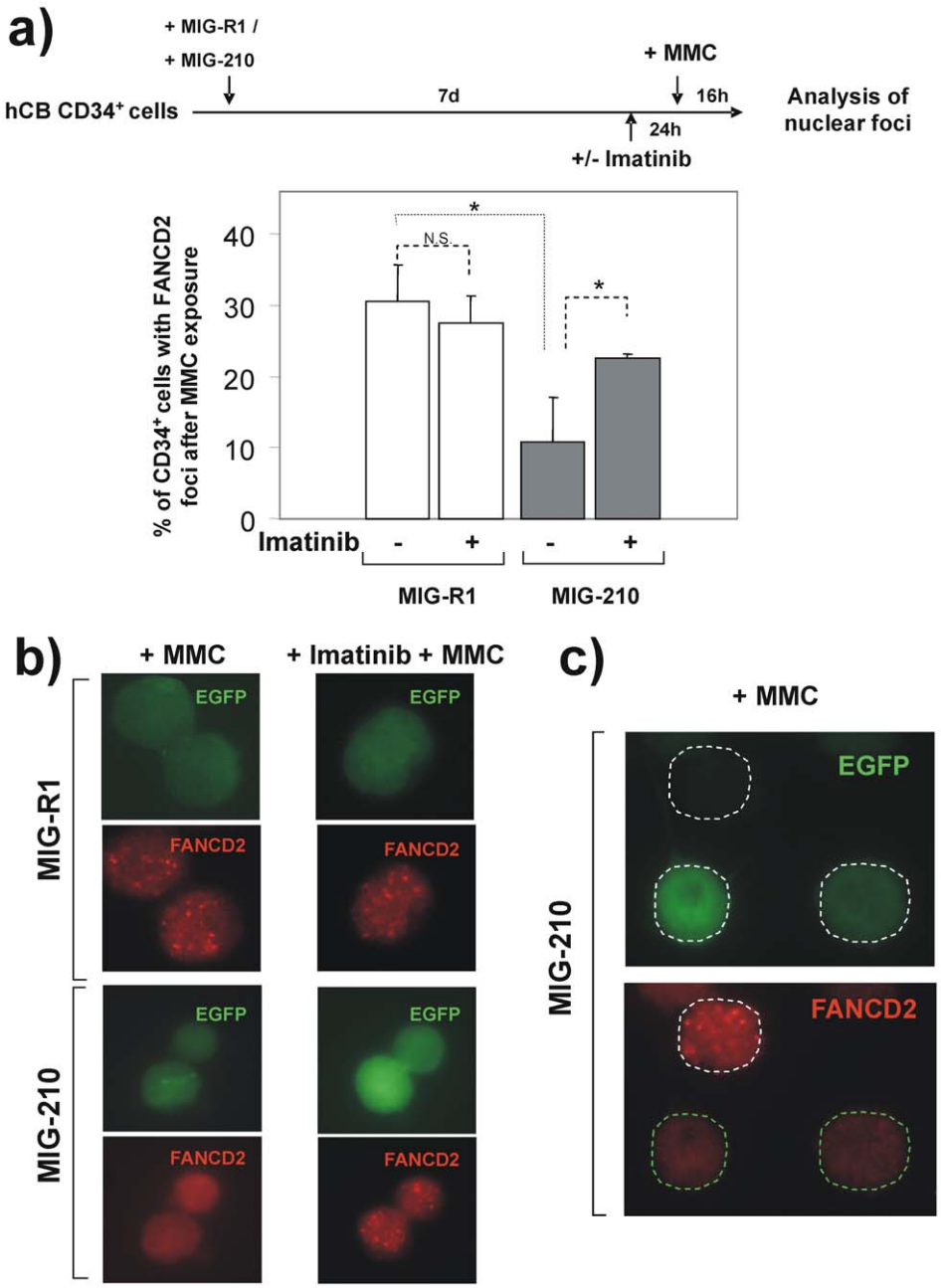

Figure 2. Direct effect of *BCR/ABL* upon the formation of nuclear FANCD2 foci in human cord blood CD34$^+$ cells. a) Experimental protocol and analysis of the proportion of cord blood CD34$^+$ cells with FANCD2 nuclear foci after transduction with retroviral vectors expressing *EGFP* (MIG-R1) or *EGFP* plus *BCR/ABL* (MIG-210). The effect of imatinib upon the formation of FANCD2 foci in these cells is also shown. In all instances cells were treated with MMC (40 nM) 16 h prior to conduct the immunofluorescence studies. Bars show mean ± s.e. of values corresponding to three independent experiments. *Differences were significant at $p<0.01$. b) Representative pictures showing restored formation of FANCD2 nuclear foci in MIG-210-transduced CD34$^+$ cells treated with imatinib. Nuclear foci were exclusively scored in cells expressing the retroviral vector marker gene (EGFP). c) Representative pictures showing the specific inhibition of FANCD2 foci in human CD34$^+$ cells transduced with MIG-210. Note that only cells expressing the marker EGFP, co-expressed with BCR/ABL in this vector, are negative for FANCD2 foci.
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fluorescent cells (thus harbouring the MIG-210 provirus) were mostly negative for FANCD2 foci (see representative pictures in Figure 2c). These results indicate that the tyrosine kinase activity of p210 is responsible of the impaired formation of nuclear FANCD2 foci in MIG-210 transduced CD34$^+$ cells.

Because the decreased proportion of MIG-210 transduced CD34$^+$ cells with FANCD2 foci could be consequence of a reduced number of cells in S-phase[25], we investigated the cell cycle distribution of MIG-R1 and MIG-210 transduced cells. As shown in Figure S1, the proportion of CD34$^+$ cells in S-phase was increased, rather than decreased, after transduction with the MIG-210 vector. Additionally, and given that FA cells are classically arrested in the G2/M phase after treatment with DNA cross-linking drugs[26], we also determined the percentage of MIG-210-transduced CD34$^+$ cells in G2/M after MMC exposure. As shown in representative histograms of Figure S1, no evident blockage in

the G2/M phase was observed in MMC-treated MIG-210-transduced $CD34^+$ cells at doses conventionally used for FA diagnosis (40 nM).

We also speculated that the inhibitory effects of BCR/ABL upon the formation of FANCD2 foci in MMC-treated cells could be due to a potentially lower generation of DSBs in *BCR/ABL* cells. To rule out this possibility, we determined the generation of DSBs in MIG-R1 and MIG-210 transduced CB $CD34^+$ cells by means of the analysis of γ-H2AX nuclear foci. A higher number of DSBs was observed in MIG-210 cells compared to MIG-R1 cells in the absence of MMC treatment (Figure S2), consistent with the reported effects of BCR/ABL on the generation of ROSs[8,9,10]. After MMC, a similar proportion of cells with γ-H2AX foci was observed in control and p210-transduced cells (Figure S2). These observations indicate that the low proportion of *BCR/ABL* cells with FANCD2 foci is not attributable to an impaired generation of DSBs.

The impaired formation of nuclear FANCD2 foci in *BCR/ABL* cells is not due to a defect in FANCD2 monoubiquitination

Since the formation of nuclear FANCD2 foci requires the monoubiquitination of this protein[27], we then investigated whether *BCR/ABL* was able to inhibit FANCD2 monoubiquitination in CB $CD34^+$ cells. Although MIG-R1 and MIG-210 vectors mediated similar transductions of CB $CD34^+$ cells (28% and 32% of $CD34^+$ cells were $EGFP^+$, respectively, at 48 h post-transduction), 91% of MIG-210 transduced cells expressed the EGFP marker 7 days after transduction, while 29% of MIG-R1 transduced cells were $EGFP^+$ at this time (Figure 3a), showing that *BCR/ABL* mediates a proliferation advantage in $CD34^+$ cells. Nuclear protein extracts from these samples were analyzed by western blot to investigate the presence of the monoubiquitinated and non-ubiquitinated FANCD2 bands. As expected, the negative control consisting of FA-A LCLs only expressed the non-ubiquitinated FANCD2 isoform (FANCD2-S). FANCD2 was, however, efficiently monoubiquitinated (FANCD2-L) not only in control $CD34^+$ cells transduced with the MIG-R1 vector, but also in MIG-210 transduced $CD34^+$ cells (see Figure 3b). These results thus indicate that BCR/ABL does not interfere with the function of the FA core complex, required for FANCD2 monoubiquitination, but rather with downstream steps in the FA pathway.

The impaired formation of FANCD2 foci in *BCR/ABL* cells can be reverted by inhibitors of the proteasome and the PI3K/Akt pathway and by the ectopic expression of BRCA1

Previous studies in healthy cells have shown that BRCA1 is required for the accumulation of FANCD2 at sites of DNA damage but not for FANCD2 monoubiquitination[27,28,29]. Given that BRCA1 levels are decreased in *BCR/ABL* cells[15], we investigated the involvement of BRCA1 in the defective capacity of *BCR/ABL* cells to generate FANCD2 foci, using two different pharmacological approaches. Because of the involvement of proteasome in reduced BRCA1 levels observed in *BCR/ABL* cells [15], the effect of a proteasome inhibitor, MG132, upon the formation of BRCA1 and FANCD2 foci was first investigated. Additionally, because the PI3K/AKT chemical inhibitor, LY294002, has been described to control BRCA1 activation in breast cancer cells [30], this inhibitor was also used in parallel to MG132. Purified MIN-210 and MIN-R1 transduced $CD34^+$ cells were treated with MMC for 16 h, and then with MG132 or LY294002 prior to conduct immunofluorescence analyses of BRCA1 and FANCD2 foci (Figure 4). As shown in this figure, the proportion of *BCR/ABL* cells with nuclear BRCA1 foci, and also of FANCD2 foci, was significantly increased when samples were treated with either of these inhibitors.

Since MG132 and LY294002 may have effects upon pathways not directly related to BRCA1, we aimed to confirm the role of BRCA1 in the impaired formation of FANCD2 foci of *BCR/ABL* cells by means of the ectopic expression of BRCA1 in these cells. To this aim, $CD34^+$ cells transduced with MIG-R1 and MIG-210 retroviral vectors were re-transduced four days afterwards with neo^r or $BRCA1/neo^r$ retroviral vectors. Co-transduced cells were then selected with geneticin for eight days, and finally exposed to MMC or maintained in MMC-free medium. Geneticin-resistant cells that expressed the EGFP protein, thus co-transduced with *BCR/ABL* and *BRCA1* vectors or their respective controls, were scored for the formation of FANCD2 and BRCA1 foci (see schematic protocol in Figure 5a). As it was observed in experiments presented in Figure 3a, the transduction of $CD34^+$ cells with MIG-210 induced a progressive expansion of transduced cells, which implied that at the end of the incubation period most cells (>90%) were $EGFP^+$. Significantly, the ectopic expression of BRCA1 in MMC-treated MIG-210 transduced cells, not only increased the formation of BRCA1 foci, but also of FANCD2 foci (see Figure 5b and representative pictures in Figure 5c).

Our data thus show that *BCR/ABL* accounts for the impaired formation of BRCA1 foci in *BCR/ABL* expressing cells, and that this effect mediates an impairment in the generation of nuclear FANCD2 foci in these cells.

The ectopic expression of BRCA1 reverts the generation of aberrant centrosomes induced by *BCR/ABL*

Because BRCA1 inhibition caused amplification and fragmentation of centrosomes in cells from mammary tissue[31], in the next set of experiments we aimed to investigate the role of BRCA1 in centrosome aberrations, characteristic of *BCR/ABL* cells[32]. To this aim, we investigated the presence of supernumerary centrosomes (more than 2 centrosomes per cell) in control $CD34^+$ cells, as well as in *BCR/ABL* $CD34^+$ cells, either re-transduced with a control Neo^r RV or with a BRCA1/Neo^r RV. The experimental protocol used in these experiments was similar to the one described in the immunofluorescence studies of Figure 5.

In contrast to control CD34+ cells, where only cells with one or two centrosomes were observed, the mere expression of the *BCR/ABL* induced multiple aberrant centrosomes in these cells as early as 9 days post-transduction (see Figure 6a and representative pictures in Figure 6b). Moreover, these experiments showed that the ectopic expression of BRCA1 in *BCR/ABL* cells reverted the generation of aberrant centrosomes induced by *BCR/ABL*. This observation demonstrates the role of disrupted pathways associated to BRCA1 down-regulation in the centrosomal instability of CML cells.

The BCR/ABL-mediated interference of the FA/BRCA pathway does not compromise cell survival to DNA cross-linking drugs though induces chromosomal instability

Because defects in the FA/BRCA pathway may compromise the survival of *BCR/ABL* cells exposed to DNA cross-linking drugs[26] in the next set of experiments we investigated the sensitivity of *BCR/ABL* and control $CD34^+$ cells to MMC. To this aim, CB $CD34^+$ cells were transduced with the MIN-210 RV and the corresponding control MIN-R1 RV. Two days after transduction, cells were subjected to immunomagnetic cell sorting and cultured in methylcellulose with increasing concentrations of MMC. Fourteen days later, colonies were scored and MMC-survival curves

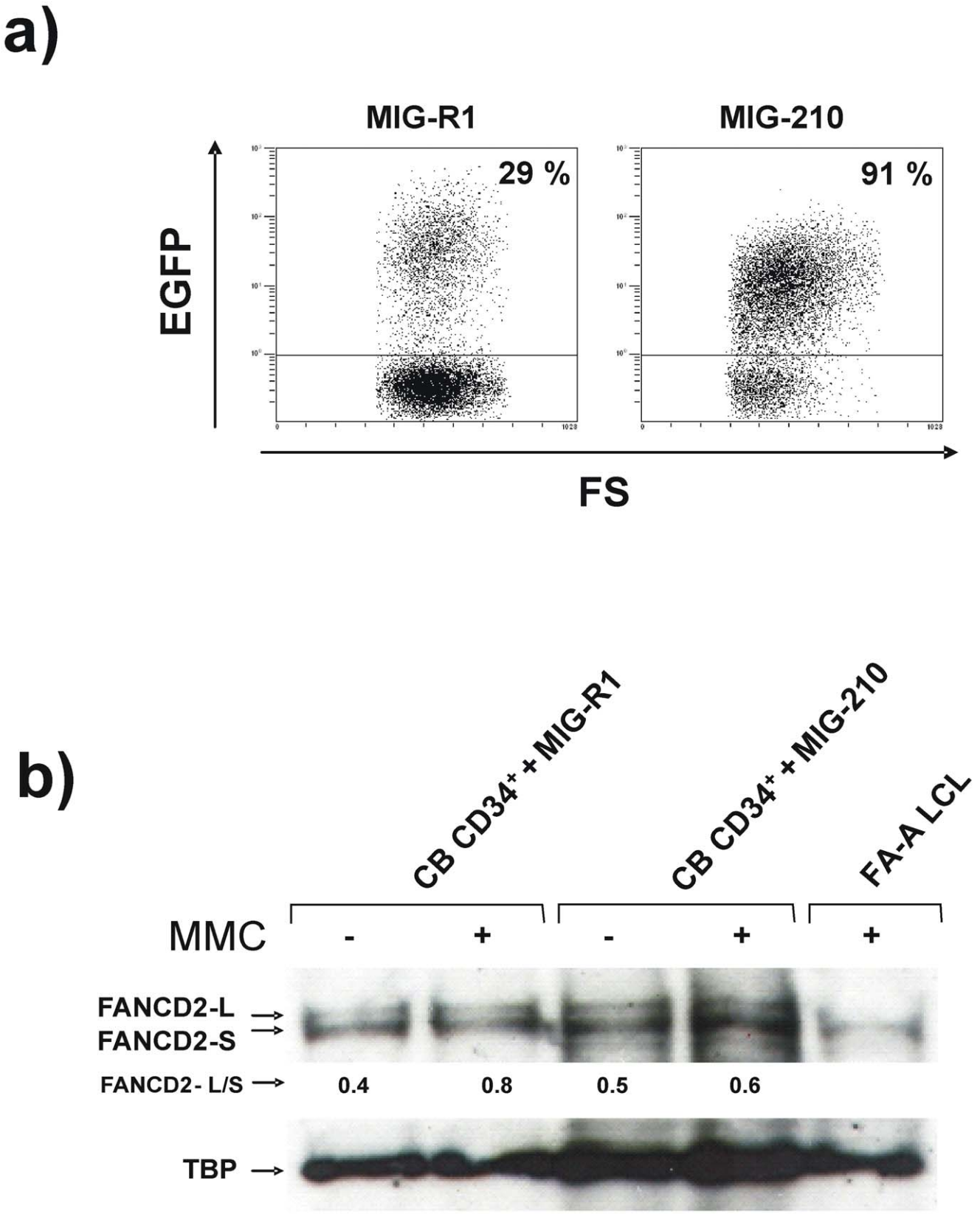


Figure 3. Efficient monoubiquitination of FANCD2 in *BCR/ABL*- transduced cord blood CD34$^+$ cells. a) Flow cytometry analysis showing the proportion of cord blood CD34$^+$ cells expressing the retroviral marker EGFP, 7 days after transduction with MIG-R1 or MIG-210 vectors. b) Western blot analysis of monoubiquitinated (FANCD2-L) and non ubiquitinated FANCD2 (FANCD2-S) in samples shown in panel a. As a negative control of FANCD2 ubiquitination, LCLs from a FA-A patient was also included. Ratios between FANCD2-L/FANCD2-S are shown.
doi:10.1371/journal.pone.0015525.g003

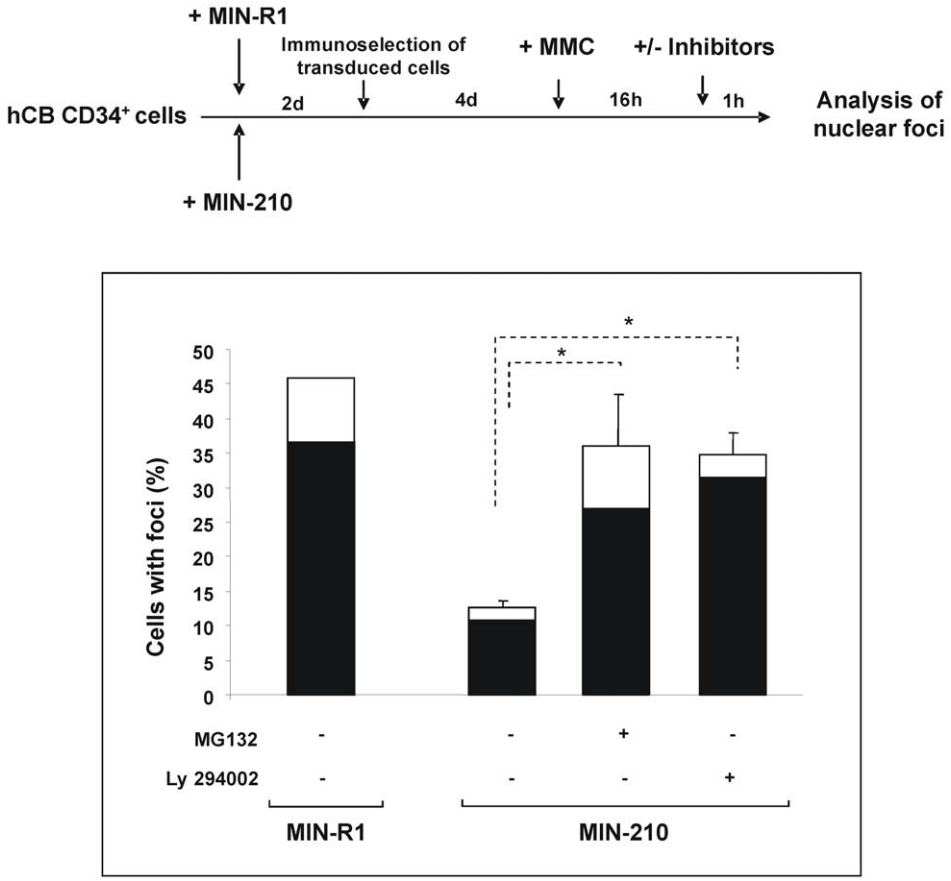


Figure 4. Reversion of the deficient formation of BRCA1 and FANCD2 foci in *BCR/ABL*-transduced cord blood CD34$^+$ cells by inhibitors of the proteasome and the PI3K/Akt pathway. Experimental protocol and analysis of the proportion of MIN-R1 and MIN-210-transduced cord blood CD34$^+$ cells with BRCA1 (white bars) or FANCD2 foci (black bars) after treatment with the proteasome inhibitor MG132, or the PI3K/AKT inhibitor Ly294002. Bars show mean ± s.e. of values corresponding to three independent experiments. *Differences were significant at $p<0.01$.
doi:10.1371/journal.pone.0015525.g004

determined[23]. As shown in Figure 7, MIN-210 transduced CD34$^+$ cells were 5-fold more resistant to MMC compared to control MIN-R1 transduced cells (IC50: 51.79±18.24 nM and 11.78±1.25 nM MMC, respectively). These results contrast to the classical MMC-hypersensitivity observed in cells with a disrupted FA/BRCA pathway, indicating that other pathways promoting cell survival are up-regulated in *BCR/ABL* cells. This observation is consistent with previous data showing the ability of BCR/ABL to interfere with cellular apoptosis pathways[33,34,35].

Finally, because cells with an impaired FA/BRCA pathway are characterized by an increased chromatid-type chromosomal instability, particularly after exposure to DNA cross-linking drugs, we investigated the spontaneous and DEB-induced chromosomal instability of CD34$^+$ cells previously transduced with the BCR/ABL RV (MIN-210) and its respective control (MIN-R1). Additionally, to test the influence of the ectopic expression of *BRCA1* on the chromosomal instability of *BCR/ABL* cells, *BCR/ABL*-transduced samples were re-transduced with control (*Neo^r*) or *BRCA1* (*BRCA1/Neo^r*) RVs, as described in materials and methods. In one experiment, chromosomal instability data was confirmed with MIG-R1 and MIG-210 RVs. Because similar data were obtained in this experiment, data in Figure 8 shows pooled results obtained with both vector families.

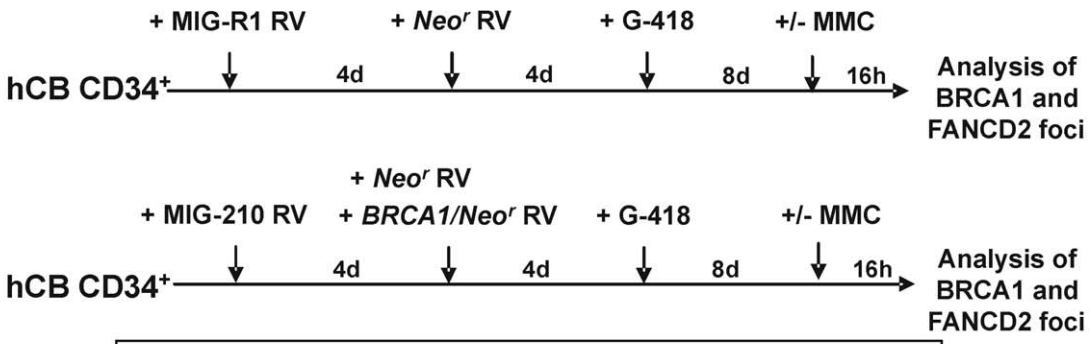
As shown in Figure 8a, a low proportion of control CD34$^+$ cells (cells transduced with MIG-R1 or MIN-R1 RVs plus the control *Neo^r* vector) either unexposed or DEB-exposed cells, contained chromosomal aberrations (5% and 7%, respectively). In no instance multiple chromosomal aberrations were observed in this control group, regardless that samples were exposed to DEB or not (Figure 8b). When CD34$^+$ cells were transduced with *BCR/ABL* RVs (plus the control *Neo^r* vector), the proportion of cells with chromosomal aberrations, specially of chromatid-type (see representative picture in Figure 8c), increased 2-fold in unexposed cells, and 3- fold in DEB-exposed cells, compared to control CD34$^+$ cells (Figure 8a). Differences were even more marked when cells with multiple chromosomal aberrations were scored, mainly after DEB exposure. In this case, almost 10% of the metaphases contained two or more aberrant chromosomes (Figure 8b). Notably, the proportion of *BCR/ABL* cells with aberrant (Figure 8a) - and more markedly with multi-aberrant chromosomes (Figure 8b) - was reduced when these cells were re-transduced with the *BRCA1/Neo^r* RV.

Taken together, these results show that the disruption of the FA/BRCA pathway in *BCR/ABL* cells mediates centrosomal amplification and chromosomal instability, and that this effect can be partially reverted by the ectopic expression of BRCA1.

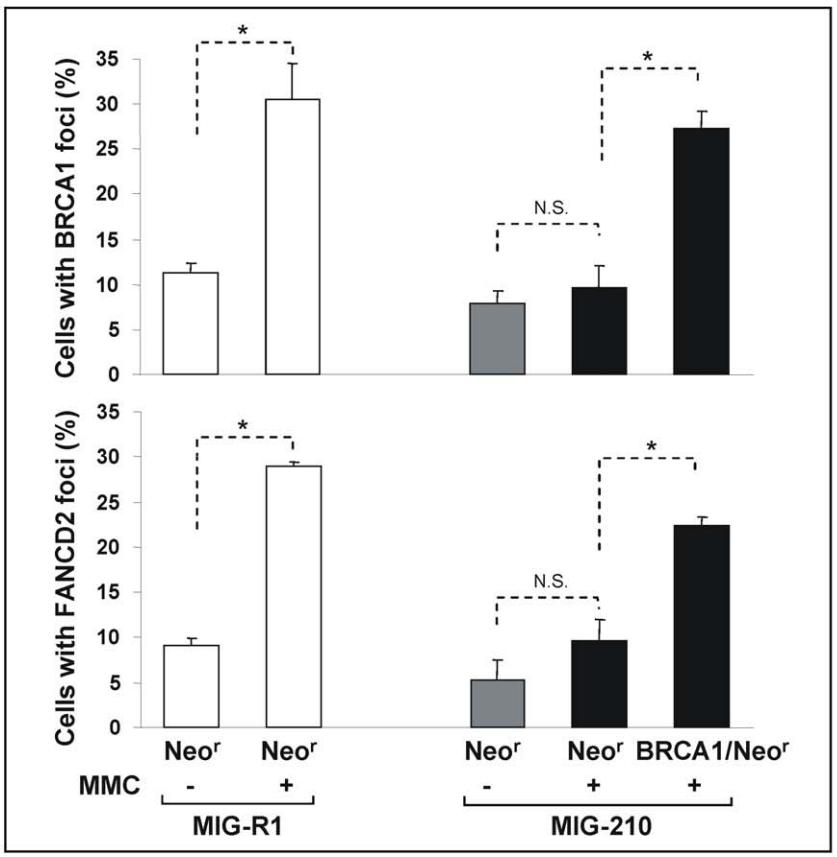
Discussion

Our study aims to offer new clues to understand the molecular pathways accounting for the genetic instability of CML cells. Our hypothesis that defects in the FA pathway may play a role

a)


+ MIG-R1 RV
+ Neor RV
+ G-418
+/- MMC
hCB CD34+
4d
4d
8d
16h
Analysis of BRCA1 and FANCD2 foci
+ Neor RV
+ MIG-210 RV
+ BRCA1/Neor RV
+ G-418
+/- MMC
hCB CD34+
4d
4d
8d
16h
Analysis of BRCA1 and FANCD2 foci

b)


Cells with BRCA1 foci (%)
Cells with FANCD2 foci (%)
35
30
25
20
15
10
5
0
*
N.S.
Neor
Neor
Neor
Neor
BRCA1/Neor
MMC
-
+
-
+
+
MIG-R1
MIG-210

c)

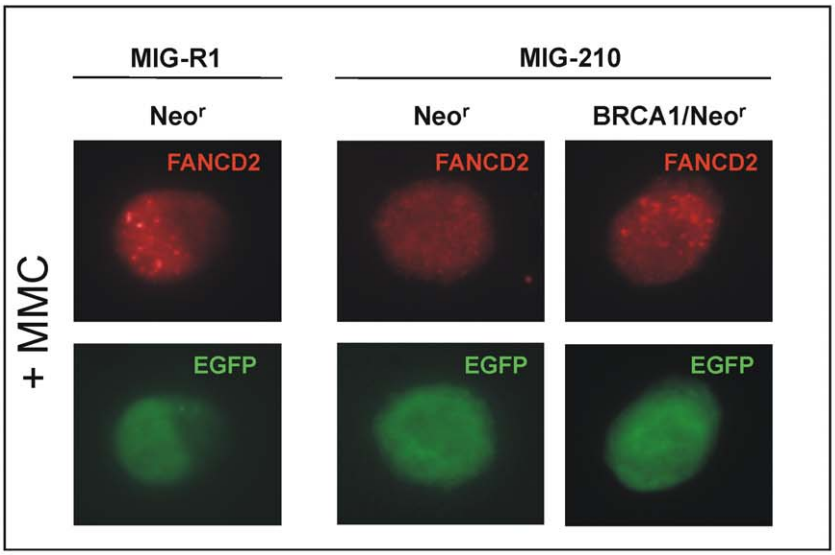

MIG-R1
MIG-210
Neor
Neor
BRCA1/Neor
FANCD2
FANCD2
FANCD2
+ MMC
EGFP
EGFP
EGFP

Figure 5. Reversion of the deficient formation of BRCA1 and FANCD2 foci in *BCR/ABL*-transduced cord blood CD34$^+$ cells by the ectopic expression of *BRCA1*. a) Experimental protocol used for investigating the effects mediated by the ectopic expression of BRCA1 upon the formation of BRCA1 and FANCD2 foci in *BCR/ABL*-transduced cells. b) Analysis of the proportion of MIG-R1 (white bars) and MIG-210 (grey and black bars) transduced cord blood CD34$^+$ cells with BRCA1 or FANCD2 foci after re-infection with vectors expressing the phosphotransferase gene (Neo^r; grey bars) or *BRCA1* plus *neo^r* (BRCA1/Neo^r; black bars). Samples were exposed to 0 or 40 nM MMC prior to analyses of nuclear foci in EGFP$^+$ cells. Bars show mean ± s.e. of values corresponding to three independent experiments. *Differences were significant at $p<0.01$. c) Representative pictures of MMC-treated cells corresponding to panel b, are shown.
doi:10.1371/journal.pone.0015525.g005

in this process derive from previous studies showing the relevance of the FA pathway to control the genomic stability of the cell[18,19] and also from observations showing genetic and epigenetic alterations of FA genes, both in inherited and acquired cancer[36,37,38,39,40,41].

In our first experiments we investigated the ability of CML cells to generate FANCD2 nuclear foci, a central process in the FA pathway (see review in[20]), both during cell proliferation and after exposure to DNA cross-linking agents. Using Mo7e-p210 and CD34$^+$ cells from CML patients, we observed that in contrast to

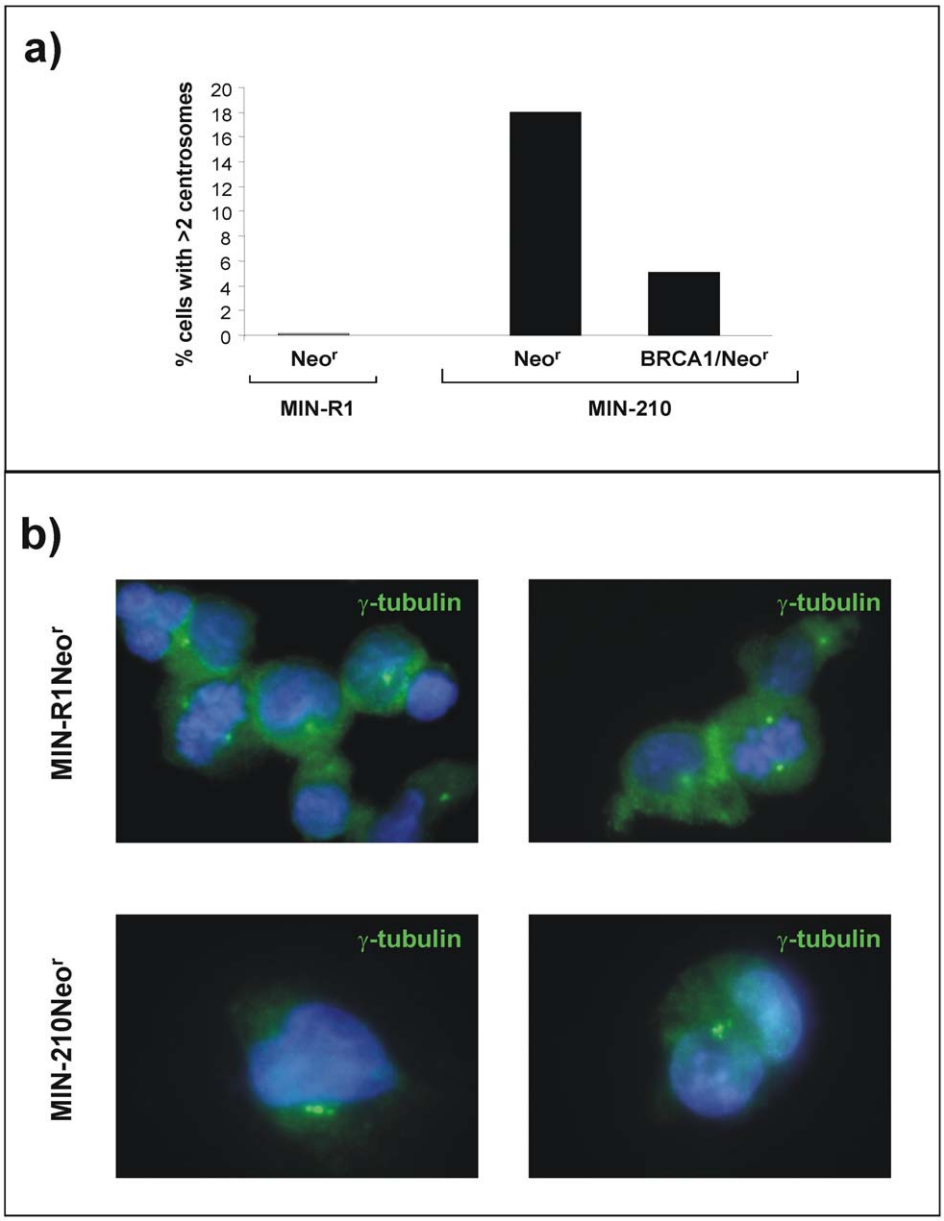


Figure 6. The ectopic expression of BRCA1 reverts the generation of aberrant centrosomes induced by *BCR/ABL*. a) Analysis of MIN-R1 or MIN-210-transduced cord blood CD34$^+$cells with supernumerary centrosomes after re-infection with either Neor or BRCA1/Neor RVs. In all instances cells were exposed to 40 nM MMC prior to analysis. Data corresponding to one representative experiment is shown. b) Representative pictures corresponding to panel a) showing supernumerary centrosomes in MIN-210 *Neo^r* compared to MIN-R1 *Neo^r* cells. To identify centrosomes γ-tubulin antibody (green) was used. DAPI staining is shown in blue.
doi:10.1371/journal.pone.0015525.g006

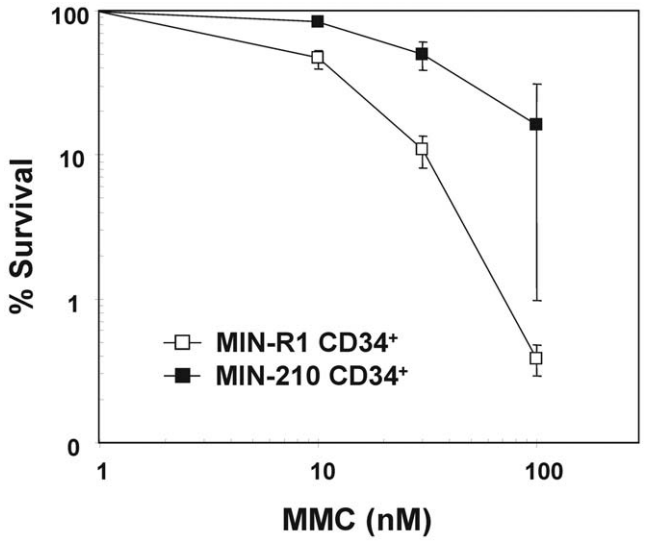


Figure 7. *BCR/ABL* induces mitomycin C resistance in cord blood progenitor cells. Cord blood CD34$^+$ cells transduced with MIN-R1 or MIN-210 RVs were purified and cultured in methylcellulose plates with increasing concentrations of MMC. Fourteen days later the total number of CFCs was scored. The graphic represents mean ± s.e of survival data obtained from three independent experiments. The IC50 value of MMC corresponding to CFCs transduced with control and *BCR/ABL* vectors was, respectively: 11.78±1.25 and 51.79±18.24 nM.
doi:10.1371/journal.pone.0015525.g007

normal cells, a very low proportion of cells harboring the *BCR/ABL* oncogene generated FANCD2 nuclear foci, even after treatment with MMC (Figure 1).

Because both the Mo7e-p210 cell line and also cells from CML patients may have accumulated secondary mutations that could account for their defective capacity to form FANCD2 foci, in subsequent experiments healthy hematopoietic progenitors consisting in CB CD34$^+$ cells transduced with vectors expressing the *BCR/ABL* oncogene were used. Previous studies have shown that human CD34$^+$ cells transduced with *BCR/ABL* vectors reproduce many of the characteristics seen in primary CML progenitors, facilitating the study of the molecular mechanisms involved in the transformation of hematopoietic precursors towards CML cells[42,43]. Our studies with human CB CD34$^+$ cells demonstrate that the mere transduction of these cells with *BCR/ABL* vectors is sufficient to inhibit the formation of FANCD2 foci, either in untreated or in MMC-treated cells (Figure 2). The relevance of the tyrosine kinase activity of BCR/ABL to inhibit the formation of FANCD2 foci was also demonstrated in these experiments by the observation that imatinib significantly restored the generation of FANCD2 foci in *BCR/ABL* cells.

Although FANCD2 monoubiquitination is required for the accumulation of FANCD2 in nuclear foci[27], our observations showing efficient FANCD2 monoubiquitination in CD34$^+$ cells transduced with the MIG-210 vector (either exposed or not to MMC; Figure 3) demonstrate that p210 does not interfere with the upstream steps of the FA pathway.

In a recent report, Koptyra *et al* observed higher levels of FANCD2 monoubiquitination in cells from CML patients and also in *BCR/ABL*-transformed cells, compared to wild type cells, and proposed that this effect could play a role in *BCR/ABL* leukemogenesis[44]. Although we cannot rule out potential effects of BCR/ABL in up-modulating the monoubiquitination of FANCD2, we propose that the most relevant effect of this oncoprotein in the FA pathway is related to the inhibited

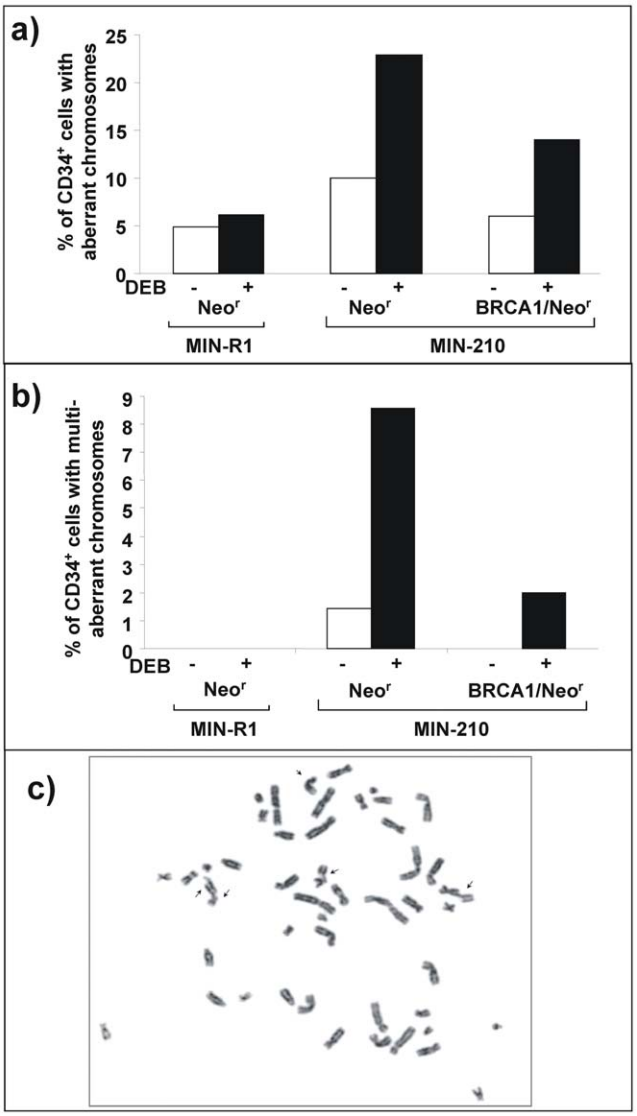


Figure 8. The ectopic expression of BRCA1 reverts the generation of chromosomal aberrations induced by *BCR/ABL*. a) Analysis of chromosomal aberrations in human cord blood CD34$^+$ cells transduced with MIN-R1 or MIN-210 and re-infected with Neo^r or *BRCA1/neo*^{r} RVs. Data corresponding to samples unexposed or exposed to DEB (0.1 μg/ml) are shown. b) Analysis of cells with multiple chromosomal aberrations in samples corresponding to panel a. Multiaberrant cells consisted on cells with two or more chromosomal breaks per cell. Data show the percentage of cells with aberrant and multiaberrant chromosomes, as deduced from the scoring of at least 50 metaphases. Pooled data obtained from two experiments with MIN RVs and one with MIG RVs are represented. c) Representative microphotograph of a multiaberrant metaphase *BCR/ABL* CD34$^+$ Neo^r in the presence of DEB. Chromatid-type aberrations are shown with arrows.
doi:10.1371/journal.pone.0015525.g008

translocation of FANCD2 to the chromatin. In this respect, different observations from other authors allowed us to hypothesize that one of the best candidates that may interfere with the translocation of FANCD2 to the nucleus of *BCR/ABL* cells was BRCA1. First, BRCA1 is post-transcriptionally down-regulated by p210[15]; second, while BRCA1 is not essential for FANCD2 monoubiquitination[28] it is required for FANCD2 binding to γH2AX at stalled replication forks[29] and for the subsequent formation of FANCD2 foci after DNA damage[27,28]; and third,

BRCA1$^{-/-}$ cells share with FA cells a chromosomal instability phenotype[45]. Additionally, because BRCA1 deficient cells have a defect in the G2/M checkpoint [45], our cell cycle studies showing that MMC-treated BCR/ABL cells are not arrested in G2/M - as it is characteristic of FA cells[26] - further suggest the role of BRCA1 in the interference of the FA pathway in these cells.

To clarify the mechanisms involved in the repression of BRCA1, and consequently in the impaired FANCD2 foci formation of CML cells, we were interested in further investigating the post-translational regulation of BRCA1 by the proteasome and the PI3K/AKT pathway, frequently activated in human cancer cells, including CML cells[46]. In this respect, data obtained in primary cells and in breast and ovarian cancer cell lines has shown that AKT1 represses BRCA1 foci formation[47,48]. Strikingly, our results show that the inhibition of PI3K/AKT pathway with LY294002 restored not only BRCA1 but also FANCD2 foci in *BCR/ABL*-transduced CD34+ cells. The same effect was observed with the proteasome inhibitor, MG132, indicating that this molecule not only restores BRCA1 expression in BCR/ABL cells, as previously described[15], but also the formation of BRCA1 and FANCD2 foci in these cells. Finally, our data in *BCR/ABL* cells co-transduced with *BRCA1*- RVs (Figure 5) confirms that the ectopic expression of BRCA1 restores, at least in part, the inhibited formation of FANCD2 foci in *BCR/ABL* cells.

As it has been previously reported, centrosome amplification occurs frequently in all types of cancer and this correlates with the malignant progression of the disease[49]. As it is the case in BRCA1-deficient cells[45], centrosome aberrations and aneuploidy are also common features of CML. In fact, previous data have shown that centrosome abnormalities correlated with the CML disease stage and preceded chromosomal aberrations in primary cells from CML patients[32]. By means of the ectopic expression of BRCA1 we show the involvement of BRCA1 in centrosomal aberrations observed in CD34$^+$ cells soon after their transduction with BCR/ABL RVs, supporting the hypothesis that this phenotype constitutes an early event in the transformation of CML cells.

The generation of centrosomal abnormalities by *BCR/ABL* is consistent with the chromosomal instability of these cells, an observation that is particularly evident after exposure to different DNA damaging agents, including oxygen species, ionizing radiation, or etoposide[10,12]. In this respect, our study shows for the first time the chromosomal instability of *BCR/ABL*-transduced CD34$^+$ cells exposed to a DNA cross-linking drug, DEB, classically used for the diagnosis of FA[50]. Strikingly, our results show that BCR/ABL confers a survival advantage, while mediates chromosomal instability to DNA cross-linking agents. The observation that BCR/ABL induces a survival advantage to MMC is, however, not surprising considering that this oncoprotein interferes with several pathways activating apoptosis[33,34,35]. In this respect, early studies showed that BCR/ABL mediates protection from DNA-damaged apoptosis in a dose-dependent manner[51], due to the capacity of the oncoprotein to regulate the expression and/or the activity of several pro- and anti-apoptotic factors signaling through the STAT5[21], PI3K/AKT[52] and RAS[53] pathways.

Finally, of particular significance was the observation that the ectopic expression of *BRCA1* in *BCR/ABL* cells markedly decreased the number of cells with aberrant, and more significantly with multi-aberrant chromosomes. Because *BRCA1* vectors also restored the formation of FANCD2 foci in *BCR/ABL* CD34$^+$ cells, our results add new insights to data previously obtained by Deutsch *et al*[15] who showed a down-regulated expression of *BRCA1* in *BCR/ABL* hematopoietic cells. In particular, our data strongly suggest that this effect interferes with the translocation of FANCD2 to sites of DNA damage, thus compromising the genomic stability of *BCR/ABL* cells.

Taken together, data obtained in this study allow us to propose that the malignant phenotype conferred by *BCR/ABL* should be at least in part related to its capacity to interfere both with downstream steps of the FA/BRCA pathway and also with other pathways with a role in apoptosis[33,34,35]. As a result of the simultaneous interference of these pathways, a survival advantage should occur in *BCR/ABL* hematopietic progenitors harboring genomic alterations which may be not compatible with the survival of untransformed cells. We therefore propose that a defective FA/BRCA pathway may contribute to the genomic instability of CML cells, thus promoting the accumulation of mutations during the progress from a chronic phase towards blast crisis.

Supporting Information

Figure S1 Cell cycle analysis of cord blood CD34$^+$ cells transduced with MIG-R1 and MIG-210 retroviral vectors. Histograms show cell cycle distributions 7 days after transduction of healthy cord blood CD34$^+$ cells with MIG-R1 or MIG-210, and exposed to 40 nM MMC (see schematic protocol in Figure 2a). At this time, more than 90% of cells exposed to the MIG-210 RV were EGFP$^+$.
(TIF)

Figure S2 Analysis of the generation of double strand breaks in cord blood CD34$^+$ cells transduced with MIG-R1 and MIG-210 retroviral vectors. The Figure shows the proportion of MIG-R1 (white bars) and MIG-210 (grey bars) transduced CD34$^+$ cells with nuclear γ-H2AX foci, both in untreated and in MMC treated (40 nM, 16 h) cells. Data from a representative experiment is shown. Representative pictures of cells with γ-H2AX foci are also shown.
(TIF)

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Author Contributions

Conceived and designed the experiments: BA FP JS JAB. Performed the experiments: AV MEA PR JAC LP RP AJ. Wrote the paper: AV MEAF PR BA FP JS JAB. Diagnosed the patients and provided samples: XA MJC. Contributed vital new reagent: HH.

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VI. ARTÍCULO 2

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LETTER TO THE EDITOR

Unraveling the role of FANCD2 in chronic myeloid leukemia

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Recent studies have offered new clues to understand the role of the Fanconi anemia (FA) pathway in the etiology and the progression of sporadic cancer (reviewed in Lyakhovich and Surralles[1] and Valeri *et al.*[2]) This is particularly true in the case of chronic myeloid leukemia (CML), where two different studies have recently shown the role of FANCD2, a critical protein in the FA pathway, in the genetic instability[3] and the leukemogenesis[4] induced by the *BCR-ABL1* oncogene.

Using human hematopoietic cells of different origin, our data showed that *BCR-ABL1* inhibits the formation of FANCD2 nuclear foci, but not the expression nor the monoubiquitination of this protein, either spontaneously or after exposure to DNA cross-linking drugs, such as mitomycin C (MMC).[3] Although the inhibited formation of FANCD2 foci in cells from CML patients or from the *BCR-ABL1*-transfected megakaryoblastic leukemia Mo7e cell line could be a consequence of secondary events related to the genetic instability of these cells, our data in fresh cord blood CD34$^+$ (CB-CD34$^+$) cells transduced with the MIN210 retroviral vector are of particular relevance, as they directly implicate the *BCR-ABL1* oncogene in the inhibited formation of FANCD2 foci in primary human hematopoietic stem/progenitor cells. In that study, we also showed that both the impaired formation of FANCD2 nuclear foci and also the centrosomal and chromosomal aberrations–measured by chromatid-type breakages–induced by *BCR-ABL1*, were significantly reverted by the ectopic expression of BRCA1, in good consistency with previous data showing a downregulated expression of BRCA1 by the *BCR-ABL1* oncoprotein.[5,6] Our results evidenced for the first time that a disruption of the FA/BRCA pathway in *BCR-ABL1* cells–in particular the impaired formation of nuclear FANCD2 foci–should have an important role in the genomic instability of CML by the co-occurrence of centrosomal amplification and DNA repair deficiencies.

In their study, Koptyra *et al.*[4] showed that in response to reactive oxygen species or MMC exposure, FANCD2-Ub was upregulated both in CD34$^+$ CML cells and in *BCR-ABL1* cell lines. These authors showed that either the inhibited expression or monoubiquitination of FANCD2 reduced the clonogenic potential of CD34$^+$ CML cells and delayed the leukemogenesis of a lymphoblastic cell line after transplantation in recipient mice. Additionally, Koptyra *et al.*[4] showed that FANCD2-Ub protects *BCR-ABL1* cells from the potential lethal effect of an excess of ROS-induced double-strand breaks (DSBs), indicating that FANCD2-Ub should have an important role in *BCR-ABL1* leukemogenesis.

In contrast to our results, Koptyra *et al.*[4] associated the upregulated expression of FANCD2-Ub in *BCR-ABL1* cells with an enhanced nuclear foci formation by FANCD2-Ub in the Mo7e cell line.[4] The authors discussed that their data were seemingly in contrast with our results, arguing that most of our measurements were performed in *BCR-ABL1*-positive CB-CD34$^+$ cells 16 h after MMC treatment. According to these authors, such time would correspond to late

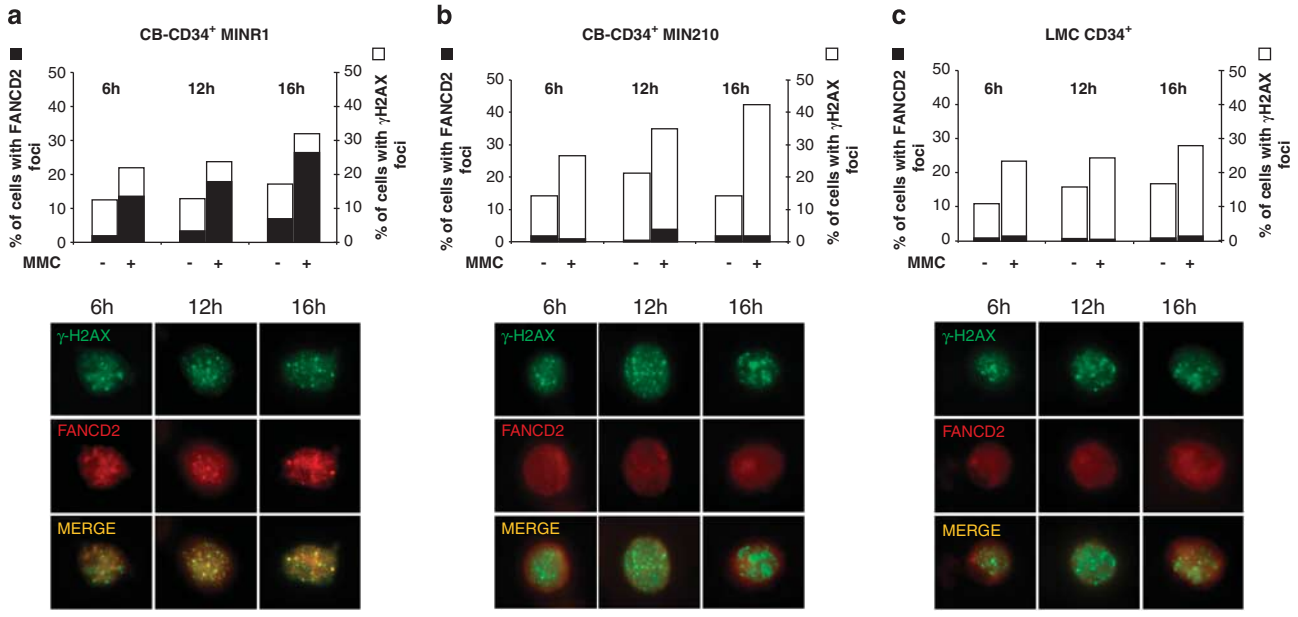

Figure 1. Kinetic analysis of γH2AX and FANCD2 nuclear foci in *BCR-ABL1*-transduced cord blood CD34$^+$ cells and in bone marrow CD34$^+$ cells from a CML patient. In (**a**, **b**) cord blood CD34$^+$ cells transduced with the control MINR1 retroviral vector (only expressing *ΔNGFR* as a marker) or with the MIN210 retroviral vector (carrying the *BCR-ABL1* and the *ΔNGFR*) were immunoselected to assure >95% transduced cells. In (**c**) fresh peripheral blood CD34$^+$ cells from a CML patient at diagnosis were used. The figure shows the proportion of cells with γH2AX and FANCD2 foci at different time points after exposure to 40 nM of MMC. In each condition, samples not exposed to MMC were also analyzed as a control. In each Figure, representative microphotographies showing nuclear γH2AX, FANCD2, and co-localized γH2AX and FANCD2 foci are included.

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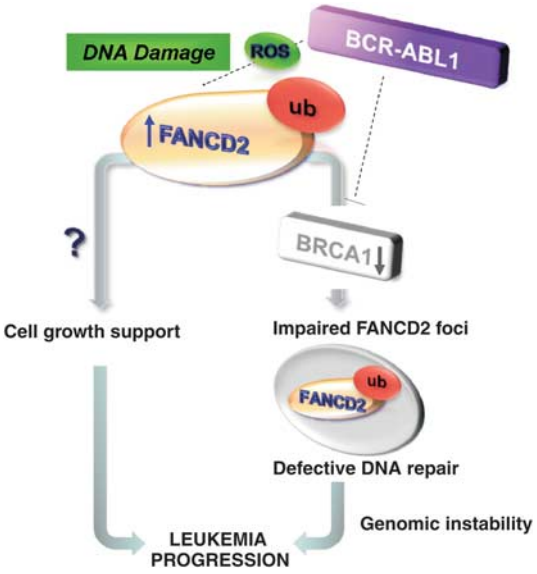

Figure 2. Proposed model of the role of FANCD2 in the leukemia progression of CML cells.

stages of DNA-damage response, where most MMC-induced DSBs would be already repaired in *BCR-ABL1*-positive cells.

Aiming to investigate whether the inhibited formation of FANCD2 foci observed in our study with *BCR-ABL1* cells could be related to the hypothesis proposed by Koptyra *et al*.,[4] the kinetics of FANCD2 and also of γH2AX (surrogate marker of DSBs) foci formation after MMC exposure was determined both in *BCR-ABL1*-transduced CB CD34$^+$ cells and also in primary CD34$^+$ cells from CML patients.

As shown in Figure 1a, a progressive increase in the percentage of control cells (CB-CD34$^+$ cells transduced with the MINR1 RV carrying the *ΔNGFR* marker) with γH2AX foci was observed after MMC exposure. A parallel kinetics was observed regarding the formation of FANCD2 foci in these cells. In CB-CD34$^+$ cells transduced with the MIN210 RV (carrying the *BCR-ABL1 plus the ΔNGFR* marker), also a gradual increase in the proportion of cells with γH2AX foci was observed (up to 16 h post MMC treatment; Figure 1b). Remarkably, most of these cells did not show FANCD2 foci (Figure 1b). Moreover, identical observations were obtained when CD34$^+$ cells from a CML patient at diagnosis were analyzed (Figure 1c).

Our data in Figure 1 demonstrate that the inhibited formation of FANCD2 foci in *BCR-ABL1* cells is occurring in cells with a high number of DSBs. This contrasts the suggestion of Koptyra *et al*.[4] who proposed that the inhibited formation of FANCD2 foci reported in our study was a consequence of conducting our analyses at late stages of DNA damage response, where most MMC-induced DSBs could be already repaired.

Because the discrepancy in FANCD2 foci formation in our study and in Koptyra's study might depend on the antibodies used for FANCD2 analyses, we investigated in healthy and also in *BCR-ABL1* CD34$^+$ cells (both *BCR-ABL1*-transduced CB CD34$^+$ cells and CD34$^+$ cells from CML patients at diagnosis) the generation of FANCD2 foci with these two antibodies. As shown in Supplementary Figure 1, almost identical results were obtained when either of these antibodies was used. In additional studies, a much higher dose of MMC (1.50 μM) as used in Koptyra's experiments was added to CD34$^+$ cells. Again, a marked inhibition in the formation of FANCD2 foci in *BCR-ABL1* cells was observed (data not shown). All these studies confirm our previous observations[3] and reinforce our main conclusion that *BCR-ABL1* inhibits the formation of FANCD2 foci, in a process that is particularly significant after DNA damage.

Taken together the results of our previous study[3] (reinforced by data from Figures 1 and Supplementary Figure 1 from this correspondence) and the results presented by Koptyra *et al*.,[4] it can be proposed that FANCD2 would have a dual role in the progression of CML (see Figure 2). Although the upregulated expression of FANCD2, followed by their monoubiquitination in K561, would facilitate the survival and/or growth of CML cells, as proposed by Koptyra *et al*,[4] the inhibited formation of FANCD2 nuclear foci by the *BCR-ABL1* oncoprotein would contribute to the genetic instability of CML cells.[3] Elucidating the pathways by which FANCD2-Ub could somehow participate in the survival/growth of CML cells independently of its chromosomal stability function–directly related to the generation of nuclear foci at sites of DNA damage–would further clarify the role of FANCD2 in the leukemia progression of CML.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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VII. ARTÍCULO 3

BCR-ABL1 cells transduced with the different anti-miR LVs or PremiR-183-96-182 cluster were incubated with colcemid (0.1 µg/ml; Gibco) for 24h. Briefly, cells were treated with hypotonic solution (0.075M KCl, Sigma), fixed in methanol:acetic acid (3:1 vol/vol), dropped onto clean slides and air-dried O.N. following standard cytogenetic procedures. Slides were stained with 10% Giemsa in phosphate buffer, pH 6.8. Chromosome aberrations including gaps, chromosome and chromatid breaks, acentric fragments, and chromosome- and chromatid-type exchanges were analyzed.

ß-galactosidase activity.

Senescence was analyzed as previously described (48). Cells positive for ß-galactosidase activity at pH6, a known characteristic of senescent cells, were counted. At least 200 cells were counted per well.

Statistical analysis.

Results are shown as the Mean± SE. Differences between groups were assessed using the Student's t-test and analyzed with Graph Pad Prism Version 5 software.

RESULTS

BCR-ABL1 mediates the up-regulation of the miR-183-96-182 cluster in CD34$^+$ cells.

Since the down-regulated expression of BRCA1 in CML cells seems to be a post-transcriptional event mediated by the BCR-ABL1 oncoprotein (2) and considering that miRNAs are important post-transcriptional regulators of gene expression, we studied the possible implication of miRNAs in BRCA1 downregulation. We focused in the so called miR-183-96-182 cluster (Figure 1a) since two members of this cluster, miR-96 and miR-183, have BRCA1 mRNA as a predicted target. Additionally, miR-182 has been implicated in BRCA1 regulation in breast cancer (30).

Expression analysis of the different miR-183 members in BM CD34$^+$ cells from healthy donors and CML patients showed a marked up-regulation of the three members of the cluster in CD34$^+$ cells from untreated CML patients at diagnosis, compared to CD34$^+$ cells from healthy donors (H.D) (Figure 1b).

Significantly, the RV-mediated over-expression of BCR-ABL1 in healthy cord blood CD34$^+$ cells (CD34:MIN210) also induced a marked up-regulation of this cluster (Figure 1C), indicating that BCR-ABL1 *per se* is involved in the over-expression of the miR-183 cluster in CML cells.

foci was observed. Notably, the overexpression of the cluster in H.D. $CD34^+$ cells did not affect the percentage of cells with foci (Figure 2d).

Since we previously demonstrated that BRCA1 downregulation in BCR-ABL1 cells abrogates the mobilization of FANCD2 to DNA repair foci, here we investigated whether this effect was reverted by the downregulation of the different miR-183 members. As expected, an evident re-expression of FAND2 foci was observed in MMC-treated BCR-ABL1 $CD34^+$ cells as a consequence of the downregulation of the different members of mir-183 cluster (Suppl Fig 4).

Because FOXO3a is regulated by the miR-183 cluster in BCR-ABL1 cells (Figure 2a), and given that this transcription factor interacts with the monoubiquitinated form of FANCD2 to detoxify ROS in the presence of H_2O_2 (43), we tested whether FANCD2 and FOXO3a interacts to detoxify spontaneous ROS generated in BCR-ABL1 cells. As shown in figure 2e in the absence of any exogenous damage, the immunoprecipitation of FOXO3a pulled-down FANCD2, preferentially the monoubiquitinated form, in BCR-ABL1 cells while this interaction was not observed in control $CD34^+$ cells. These results suggest that FANCD2 is collaborating with FOXO3a in the ROS detoxification in BCR-ABL cells. To further investigate whether the modification of the miR-183 cluster expression could also regulate this interaction, we conducted FANCD2 and FOXO3a immunoprecipitation assays in BCR-ABL1 $CD34^+$ cells transduced either with the different Anti-miR or with the PremiR cluster. As shown in Figure 2f, when cells were transduced with Anti-miR-96, -182 or -183, FANCD2 the interaction between FANCD2 and FOXO3a was maintained. However, the re-expression of this cluster abolished this interaction, probably due to the abrogation of FOXO3a in these cells (Figure 2f).

Regulation of DNA damage response by miR-183-96-182 cluster in BCR-ABL1 cells.

Since the interaction between FOXO3a and FANCD2 is responsible of ROS detoxification, the knock-down of the different members of the cluster reduced the superoxide content in the BCR-ABL1 cells (Figure 3a). On the contrary, overexpression of miR-183-96-182 cluster, that almost completely abrogates FOXO3a, leads to a significant increase in superoxide anion levels (Figure 3a).

To measure the induction of DNA damage, in terms of DSBs, we analyzed the activation of H2AX, as a DNA damage sensor. While the downregulation of the different members of the miR183 cluster did not modify the generation of γH2AX, an unexpected significant reduction in the percentage of γH2AX-positive cells was observed in BCR-ABL1 cells overexpressing the miR-183 cluster (Figure 3b). Strikingly,

tested in methylcellulose cultures. As shown in Figure 4, a mild decrease in the number of hematopoietic colonies was observed when BCR-ABL1 cells were transduced with the different Anti-miR-182 and Anti-miR-183 (Figure 4a and figure 4b). A similar moderate effect was observed when the three Anti-miRNAs LVs were combined (Figure 4a). When control $CD34^+$ cells were treated under identical conditions no changes in the clonogenic potential of these cells were noted in any case (Figure 4c).

Strikingly, when BCR-ABL1 $CD34^+$ cells were transduced with the PremiR-183-96-182cluster LV, a marked decrease in the number and size of hematopoietic colonies was observed (Figure 5a left panel and 5b) while clonogenicity was not affected in healthy $CD34^+$ cells (figure 5a right panel). Consistent with these studies, the *ex vivo* expansion of BCR-ABL1 cells was markedly inhibited after transduction with the PremiR-183-96-182 cluster LV, while no effects were again observed when healthy CB $CD34^+$ cells (Figure 5c).

To identify the origin of the reduced expansion capacity we analyzed the proliferative index and apoptotic rate of BCR-ABL1 cells overexpressing the PremiR-183-96-182 cluster. A significant reduction in Ki67-positive cells (Figure 5d) and also a significant increase in Annexin-V staining (Figure 5e) was noted, accounting for the reduced growth potential of these cells.

This set of experiments demonstrates that the proliferation potential of BCR-ABL1 progenitor cells is strongly and specifically inhibited when the PremiR-183-96-182cluster is over-expressed.

Pre-miR-183-96-182 cluster induces senescence in BCR-ABL1 $CD34^+$ cells.

Since the miR-183 cluster was previously linked to increased stress induced premature senescence (SIPS) (52) we measured the ß-galactosidase activity in BCR-ABL1 $CD34^+$ cells transduced with the control LV or the PremiR-183-96-182cluster LV. As shown in figure 6a and b, a significant increase in cell senescence, measured by ß-galactosidase activity, was associated to transduction with the Premi-183-96-182 LV. This observation was confirmed by HP1-γ staining, a marker of senescence-associated heterochromatin (53) detected in SIPS (54) (Supplem. Figure 7a), suggesting that increased senescence could also account for the reduced clonogenic capacity of hematopoietic progenitors. Oncogene induced-senescence (OIS) is characterized by hyperproliferation that triggers the activation of an ATR/ATM dependent DDR, resulting in the formation of γ-H2AX positive senescence-associated heterochromatin foci (SAHF) (55). However, our flow cytometry analysis indicated that H2AX phosphorylation was reduced in BCR-ABL1 cells transduced with the PremiR-183-96-182cluster LV. To solve this controversy we analyzed γ-H2AX by immunofluorescence

These results demonstrate that overexpression of miR-183 cluster in BCR-ABL1 cells resembles all the characteristics of PICS senescence and not the typical OIS senescence exhibited by oncogenic forms of RAS (58) or BRAF (59).

In a final set of experiments we investigated the possible mechanisms involved in the downregulation of PTEN in BCR-ABL1 cells.

To analyze whether *PTEN* is a direct target of miR-183 we conducted luciferase assays using the 3'UTR region of *PTEN* (siCHECK2:PTEN) and its corresponding mutated version (siCHECK2:mut PTEN). Our data showed that although miR-183 can regulate PTEN expression in BCR-ABL1 cells, there is no direct interaction between the PremiR-183-96-182cluster and *PTEN* (Supplem. Figure 9a). Considering that the miR-183 cluster regulates BRCA1 in BCR-ABL cells, we wanted to test whether the indirect regulation of PTEN by the miR-183 cluster was mediated by BRCA1. For this purpose we analyzed the expression of PTEN in $CD34^+$ control and BCR-ABL1 cells transduced either with a control vector (INEO) or a vector expressing a *BRCA1* form not recognized by miR-183 cluster. As shown in Supplem. Figure 9b the re-expression of *BRCA1* in BCR-ABL1 cells increased PTEN protein levels, suggesting a link between the miR-183 cluster, BRCA1 and PTEN. Since BRCA1 interacts with chromatin modifiers as HDACs (60) and SW1/SNF complex (61) we analyzed the regulation of *PTEN* promoter by acetylation or methylation. However, we did not observe differences in the samples from BCR-ABL1 cells (Supplementary Figure 9c and 9d).

In view of the fact that miR-183 directly regulates *EGR1* (57), a well-known transcription factor of PTEN (62, 63) we confirmed that EGR1 is upregulated in BCR-ABL1 cells when BRCA1 is overexpressed (Supplem.Figure 9c), suggesting that the downregulation of PTEN in BCR-ABL1 cells can be mediated by the reduction of EGR1 induced, both by overexpression of miR-183 cluster and the consequent downregulation of BRCA1.

DISCUSSION

Here we describe a new crosstalk signal involved in the post-transcriptional regulation of different master suppressors and we propose that genomic instability in CML results from a combination of ROS oxidative damage and inefficient FA/BRCA repair mechanism mediated by miR-183 cluster signaling. Breast cancer onset-1 (BRCA1), is a multifunctional protein that impacts in DDR response participating in homology-directed DNA repair (64), in the choice of DSB repair pathway during the end processing (65, 66), controls all the DNA damage activated checkpoints (60, 67, 68)

Taking into account the relevance of BRCA1 expression in the genomic stability in CML cells (4) our results demonstrate that up-regulation of this cluster in BCR-ABL1 cells acts as an important repressor of BRCA1 and consequently increase chromosomal instability, probably contributing to the progression of the disease. As we previously demonstrated (4) the re-expression of BRCA1, in this case by the inhibition of miR-183 cluster, also restored the mobilization of FANCD2 to DNA repair foci, confirming the role of miR-183 cluster in FA pathway control.

Although it has been previously published that BCR-ABL1 stimulates all the DSBs DNA repair pathways (HDR, and the error-prone NHEJ and SSA reviewed in (73)), resulting in unfaithful/inefficient repair and chromosomal instability, a recent study by Chakraborty et al.(74) provided conclusive proof of chromosomal instability after DNA damage in Chronic Phase CML cells. This occurs at the expense of dysregulated higher levels of classic NHEJ, that finally generates anaphase bridges during mitosis. Therefore, this CIN is thought to occur as a consequence of continuous cycles of breakage-fusion-bridge (BFB) mechanism. It is tempting to speculate a possible contribution of FA/BRCA pathway to BFB cycles also in ROS spontaneous DSBs, because of its role in unbalanced DSB repair with active suppression of NHEJ factors (75, 76) and promotion of HDR (77), together with its well-known function during mitosis, counteracting anaphase bridges formation (78) and cytokinesis failure (79). In fact, low levels of BRCA1 detected in CML cells could provoke the induction of a toxic NHEJ during replication DSB repair, because they cannot compete with 53BP1-RIF1 complex during the processing of the DSB end (80, 81). This process could allow the creation of chromosomal fusions that, throughout mitosis result in binucleated cells and micronuclei appearance and a new round of chromatid and chromosomal breakages. This is consistent with our results showing no changes in total DSB balance, measured by γ-H2AX, but an increase in chromosomal stability when we interfered miR-183 cluster and reflects that chromosomal instability is mainly caused by the fidelity in the DNA repair mechanism selected, more than the quantity of DSBs repaired. In fact, percentage of 53BP1 positive cells in S phase is markedly reduced when we interfered miR-183 cluster, suggesting that toxic NHEJ is controlled. This is also on line with our data showing that BRCA1 abrogation, due to miR-183 cluster over-expression, increased the percentage of multipolar anaphases and bi- or multinucleated cells, finally, compromising the survival or inducing senescence in these cells.

Altogether, our results demonstrate for the first time the implication of the different members of this cluster as a new mechanism involved in BRCA1 down-regulation and in the chromosomal instability of BCR-ABL1 cells.

but the role of senescence in this response has not yet been demonstrated (93-95). In our study we link for the first time the PTEN loss with senescence activation in human BCR-ABL1 hematopoietic progenitor cells. The mechanism underlying this PICS induction could be mediated by STAT5/miR183 cluster induction/PTEN-loss. Interestingly, Nogueira et al. showed that AKT activation, which is known to be activated by PTEN-loss (96), inhibits FOXO3a and the consequent accumulation of ROS, which plays a role in senescence trigger. The description in our experiments that miR-183 cluster regulates both *PTEN* and *FOXO3a*, leading to the accumulation of ROS, emerge as a new pathway in senescence activation.

Although this response can contribute to the oncogenic induced senescence of BCR-ABL1 in early stages of the disease, we demonstrate that this signal can be enhanced by the overexpression of miR-183 cluster. The fact that either the inhibition or overexpression of miR183 cluster act cytostatically in BCR-ABL1 cells suggests that PTEN requires a fine-tuning regulation as previously postulated (97).

In CML stem cells PTEN is down-regulated and behaves as a tumor suppressor (98). The same authors have proposed that the lipoxygenase ALOX5 could repress PTEN in CML (99), but this has not been yet demonstrated. However, our results confirm that the miR-183 cluster and BRCA1 modulate the PTEN levels, probably through EGR1 induction. This also indicates that EGR1 could act as a tumor suppressor in CML as was described in preliminary results from Hoffman´s lab(100).

Notably, despite the oncogenic potential of miR-183 cluster in BCR-ABL1 cells overexpression of this cluster can have a therapeutic potential either by inducing apoptosis or PTEN-loss induced senescence. Pro-senescence therapy has been recently described as a potential therapeutic strategy to combat tumorigenesis (55). In particular PICS, from a therapeutic perspective, has been proposed that offers several advantages over the induction of common oncogene induced-senescence. First, do not requires hyperproliferative burst and a DDR, therefore, is activated in shorter time avoiding genomic instability and the risk of acquiring secondary mutations. Second, the absence of DNA replication suggests that can be induced in quiescent cell types , such as cancer initiating cells, which is highly relevant in CML. Third, as PICS is a p53-driven response and its activation is predominantly translational, this implies a potential to firing levels of p53 targeting HDM2. According with our results, a few issues need to be taken into consideration. Although DDR is not activated in PICS we have observed DNA damage, measured by 8-oxodG, and localization of 53BP1 at DNA repair *foci*. This could lead to new mutations that could contribute to escape from senescence or, in view of PICS being a p53-dependent pathway, the appearance of a selective pressure for the loss of p53 function, commonly mutated in advanced stages of

FIGURES

Figure 1: miR-183-96-182cluster is up-regulated in BCR-ABL1 transduced CD34$^+$ cells and CML CD34$^+$ samples. **a)** Schematic representation of miR-183-96-182 cluster and the sequence of miR-96, miR-182 and miR-183. The seed region is shown in red. **b)** Analysis of miR-183-96-182 cluster expression in CD34$^+$ cells obtained from the bone marrow of five healthy donors (H.D.) and five newly diagnosed CML patients. **c)** Relative expression of miR-96, miR-182 and miR-183 in CD34$^+$ cells transduced with a control vector (white bars) or a BCR-ABL1 RV (black bars) and immunoselected (see material methods).
Expression level is shown as ratio between each miRNA and *RNU6B* gene and normalized to mean values corresponding to H.D. samples (panel b) and CD34+ control cells (panel c).

Figure 2: miR-183 cluster targets BRCA1 and FOXO3a in BCR-ABL1 cells. a) Analysis by westernblot of BRCA1 and FOXO3a in cells transduced with the different Anti-miR and PremiR-183 cluster. β-ACTIN was used as a loading control. **b)** The percentage of cells with BRCA1 foci was analyzed in BCR-ABL1 CD34$^+$ cells transduced with Anti-miR control LV, AntimiR-96 LV; AntimiR-182 LV or AntimiR-183 LV (left graphic), three days after transduction. Cells were incubated for 16h with 40nM of MMC, mean ± SE of four different experiments is shown. To confirm previous results, an additional experiment was conducted transfecting (Transf.) a control Anti-miR or the three Anti-miR (-96,-182 and 183) (right graphic). In all instances 200 cells were scored for each condition. **c)** Microphotographies corresponding to the different conditions used in Figure 2c, left panel. **d)** BRCA1 foci formation in BCR-ABL1 CD34$^+$ cells (left panel, black bars) or control CD34$^+$ cells (right panel, white bars) transduced with a control LV or PremiR-96,182,183 LV after DNA damage. **e)** FOXO3a immunoprecipitation in control and BCR-ABL1 cells followed by immunoblotting using antibodies against FANCD2, FOXO3a and isotype IgG heavy chain (loading control). **f)** Pull-down of FOXO3a in BCR-ABL1 cells transduced with the different Anti-miRs and the PremiR-96,182,183 vector. Immunoblotting analysis was conducted using antibodies against FANCD2 and FOXO3a. IgG heavy chain was used as a loading control. Upper panel: input. Lower panel: FOXO3a pulled-down.

Figure 3: ROS production and DDR in BCR-ABL1 cells transduced with the different Anti-miR and the Pre-miR 183-96-182 cluster. a) ROS production was measured in BCR-ABL1 cells transduced with the different vectors three days after

b) Microphotographies of hematopoietic progenitors corresponding to **panel a** left. **c)** *In vitro* proliferation of BCR-ABL1 (upper panel) and MINR1 control cells (lower panel) transduced with a control vector (white diamonds) or the Pre-miR-183-96-182 cluster (filled circles). **d)** Immunofluorescence staining with the proliferation marker Ki67 (Red) in control and BCR-ABL1 cells after seven days in culture. Mean ± SE of three different experiments is shown on the right. e) Apoptosis was evaluated by staining with Annexin-V and Dapi. The graphic represents the increase of cells positive for Annexin-V with the different treatments in comparison with BCR-ABL1 cells transduced with the Anti-miR-control. Mean ± SE of four different experiments is shown.

Figure 6: Overexpression of PremiR-183-miR-96-miR-182 in BCR-ABL1 cells induces PTEN-loss induced senescence. a) β-galactosidase activity in BCR-ABL1 cells transduced with the control vector or the PremiR-183-96-182 cluster seven days after transduction. Mean ± SE of four different experiments is shown. **b)** Representative microphotographies of panel a. **c)** Dual immunofluorescence for HP1-γ (red) and γ-H2AX (blue) in BCR-ABL1 cells transduced with the control or the Pre-miR-183-96-182 LVs. **d)** Coimmunostaining of BCR-ABL1 cells overexpressing the Pre-miR-183-96-182 or a control vector with HP1-γ (red) and 8-oxodG (blue). EGFP positive cells are shown in green. DAPI was used to counterstain the nucleus of the cells. **e)** PTEN westernblot in cells transduced with the different Anti-miR and the Pre-miR-183-96-182 cluster. β-ACTIN was used as a loading control. Ratio between PTEN and β-ACTIN is shown. **f)** Westernblot analysis to identify possible effectors of PICS: p27Kip1; p21; p53; HDM2; p16INK4A; and Phospho-p70 S6 kinase. *Unspecific band. β-ACTIN was used to normalize the level of expression of the different proteins. Ratio between protein/β-ACTIN is shown in each immunoblotting.

Supplementary Figure 1: STAT5 regulates the expression of miR-183 cluster in BCR-ABL1 cells. Expression by qRT-PCR of the different members of the cluster in comparison with *RNU6* gene was analyzed in BCR-ABL1 cells after incubating for 72h in the presence of a vehicle (white bars) or 300 µM of nicotinyl hydrazone (black bars), an STAT5 inhibitor. Data was normalized to control $CD34^+$ cells (MINR1).

Supplementary Figure 2: Transduction efficiency in BCR-ABL1 cells of the different Anti-miRs LV and Pre-miR LV measured by EGFP expression by flow cytometry.

Supplementary Figure 8: DNA damage is sensed by 53BP1 foci but not by H2AX phosphorylation in BCR-ABL1 cells overexpressing PremiR-183 cluster. a) 53BP1 (red) is also shown in control BCR-ABL1 (left panels) and Pre-miR-183-96-182 BCR-ABL1 cells (right panels). EGFP positive cells are shown in green. DAPI was used to counterstain the nucleus of the cells. Analysis was conducted seven days after transduction.

Supplementary Figure 9: Regulation of PTEN by miR-183 cluster. a) RKO cells were stably transduced with a Pre-miR-183-96-182 cluster LV and transfected either with transfected siCHECK2:PTEN or siCHECK2: mutPTEN The normalized *Renilla* luciferase activity is shown. Bars show mean values ± SE corresponding to 3 independent experiments. **b)** Control $CD34^{+}$ cells and BCR-ABL1 cells were re-transduced either with a control RV:INEO or RV:BRCA1INEO. Expression of PTEN was analyzed in these cells by westernblot. Ratio between PTEN expression and β-ACTIN is shown. **c)** Acetylation levels of Histone 3 in the promoter of *PTEN* analysed by ChIP. **d)** DNA methylation status of 18 different CpG islands of *PTEN* promoter analysed by pyrosequencing. Heat map denotes methylation grade. **e)** The level of EGR1 was analyzed by westernblot in the same treatments that in panel **b**. Ratio between EGR1 and β-ACTIN is shown.

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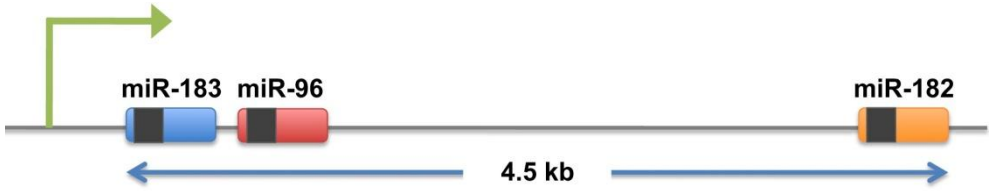
Figure 1

a)

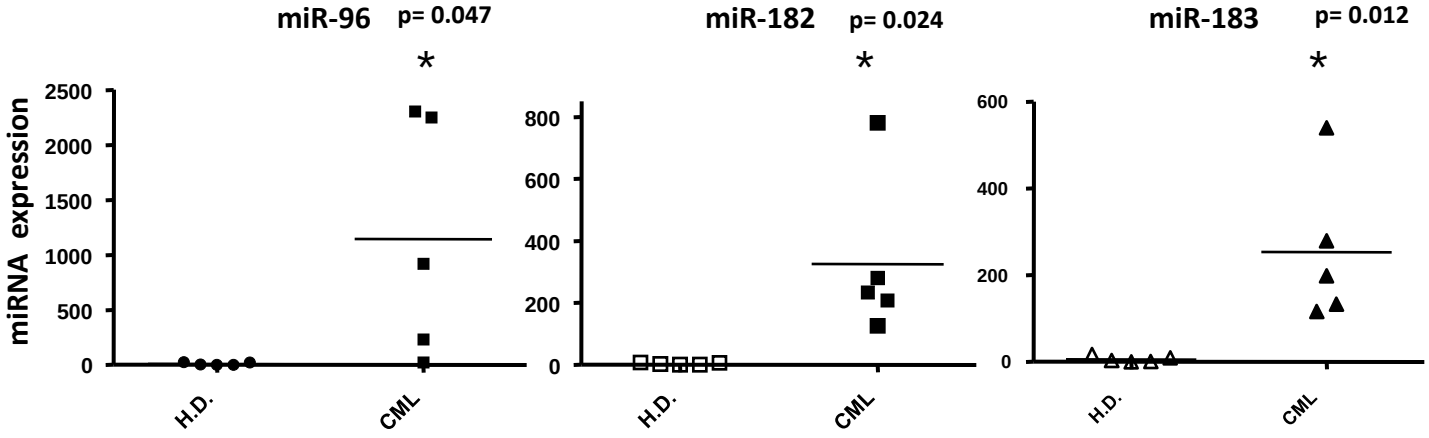


hsa-miR-96 UUUGGCACUAGCACAUUUUUGCU

hsa-miR-182 UUUGGCAAUGGUAGAACUCACACU

hsa-miR-183 UAUGGCACUGGUAGAAUUCACU

b)



c)

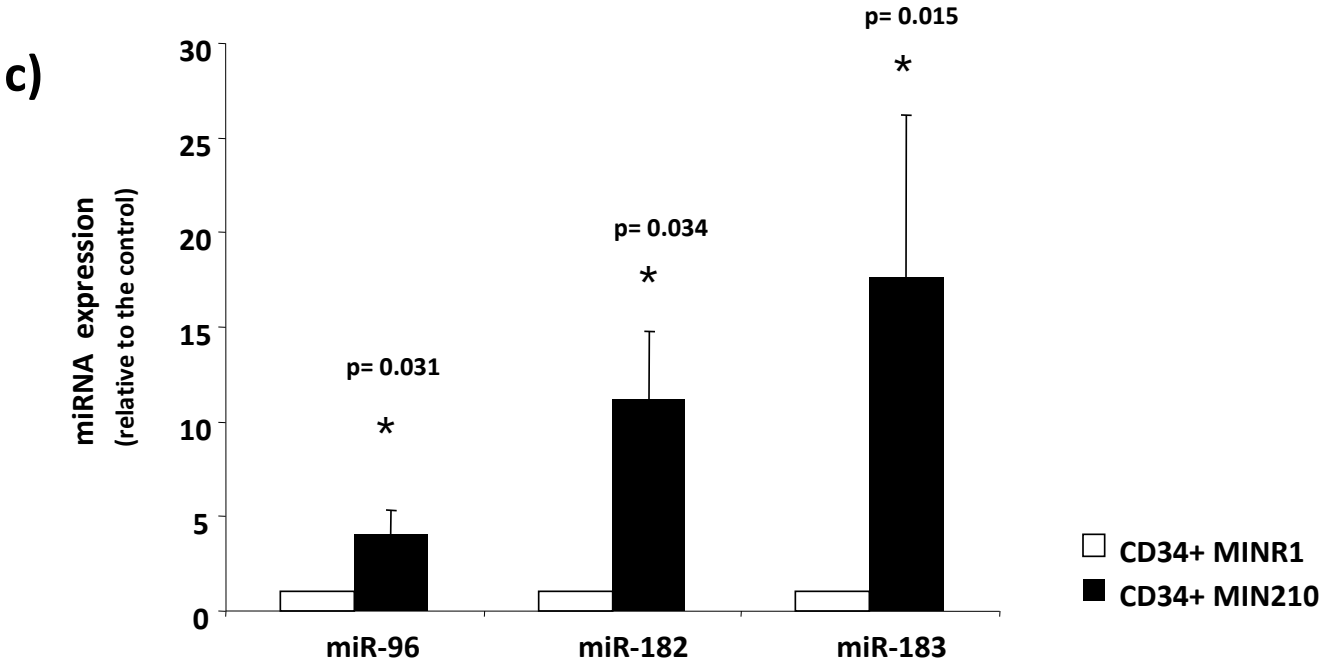
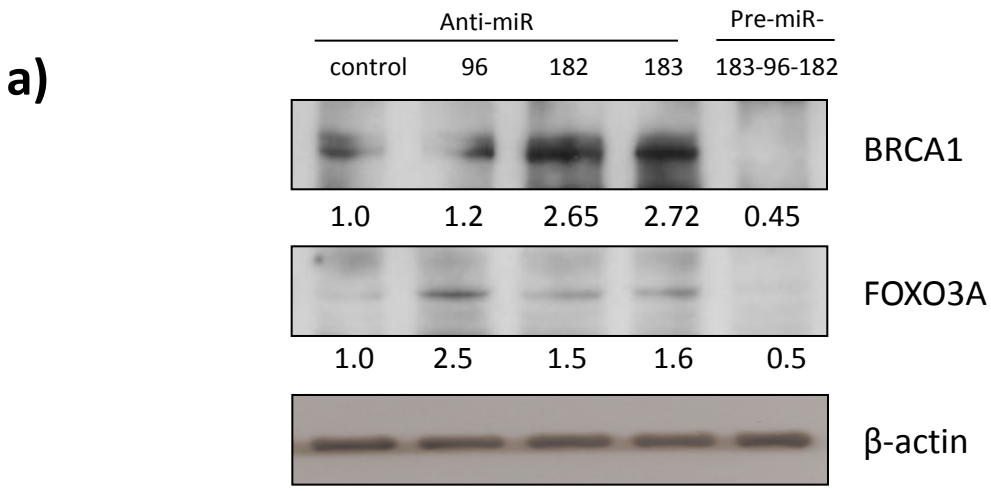
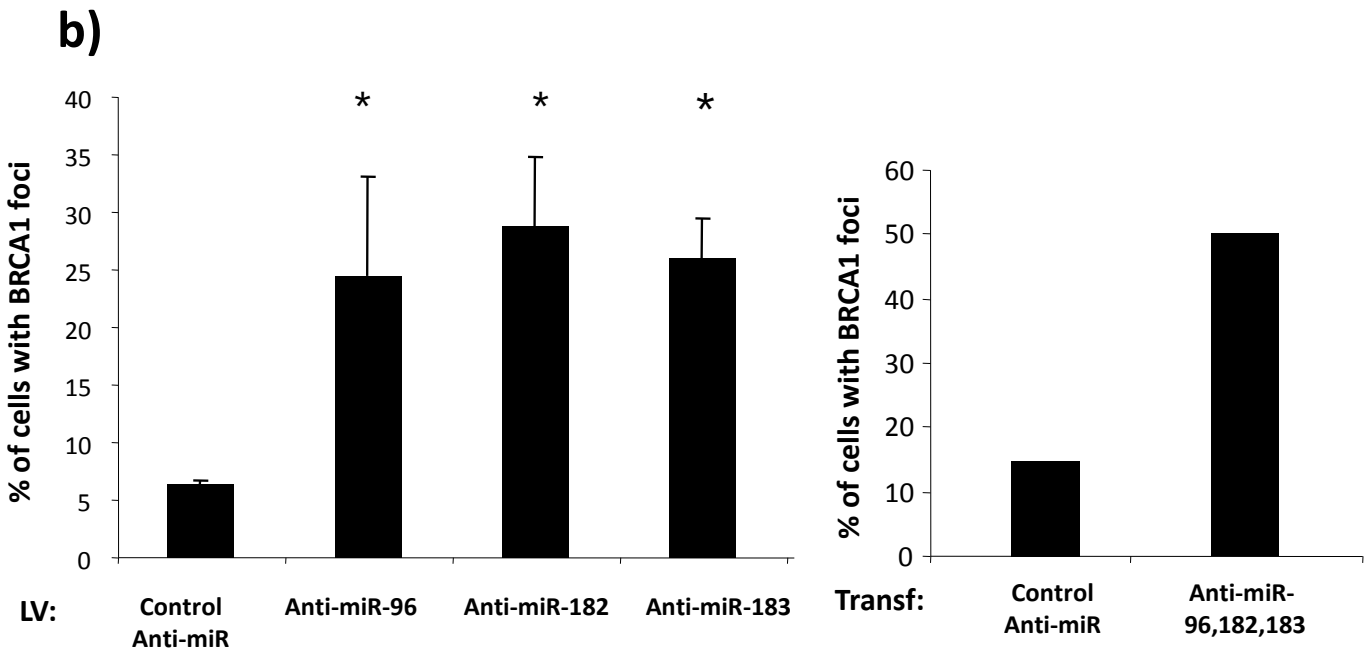


Figure 2


a)
Anti-miR
Pre-miR-
control
96
182
183
183-96-182
BRCA1
1.0
1.2
2.65
2.72
0.45
FOXO3A
1.0
2.5
1.5
1.6
0.5
β-actin


b)
% of cells with BRCA1 foci
40
35
30
25
20
15
10
5
0
*
*
*
LV:
Control Anti-miR
Anti-miR-96
Anti-miR-182
Anti-miR-183
% of cells with BRCA1 foci
60
50
40
30
20
10
0
Transf:
Control Anti-miR
Anti-miR-96,182,183

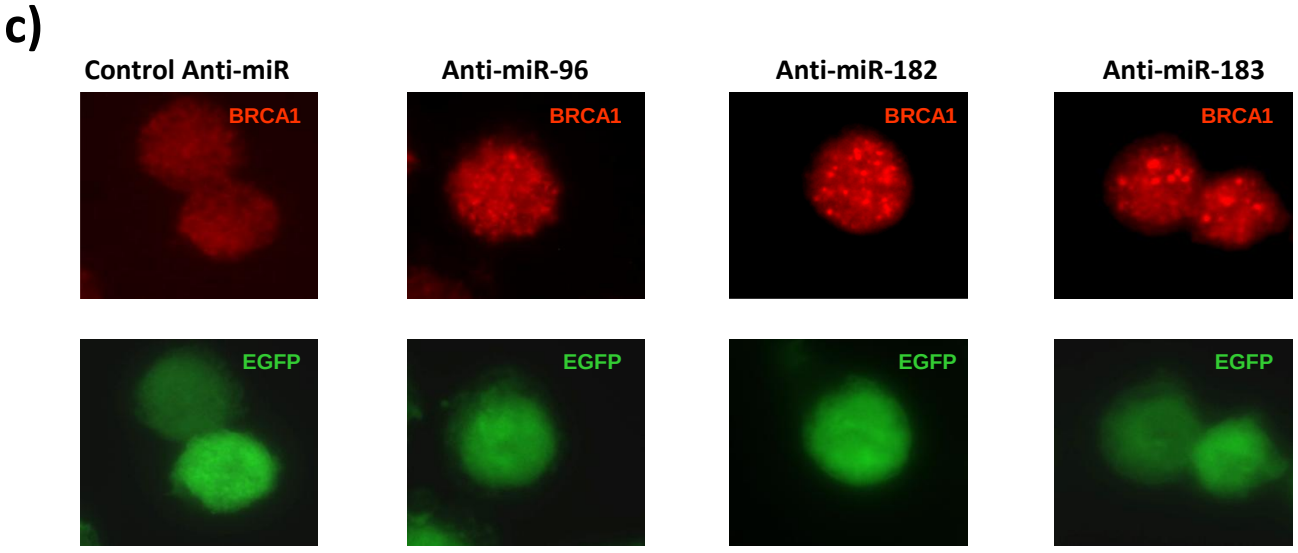
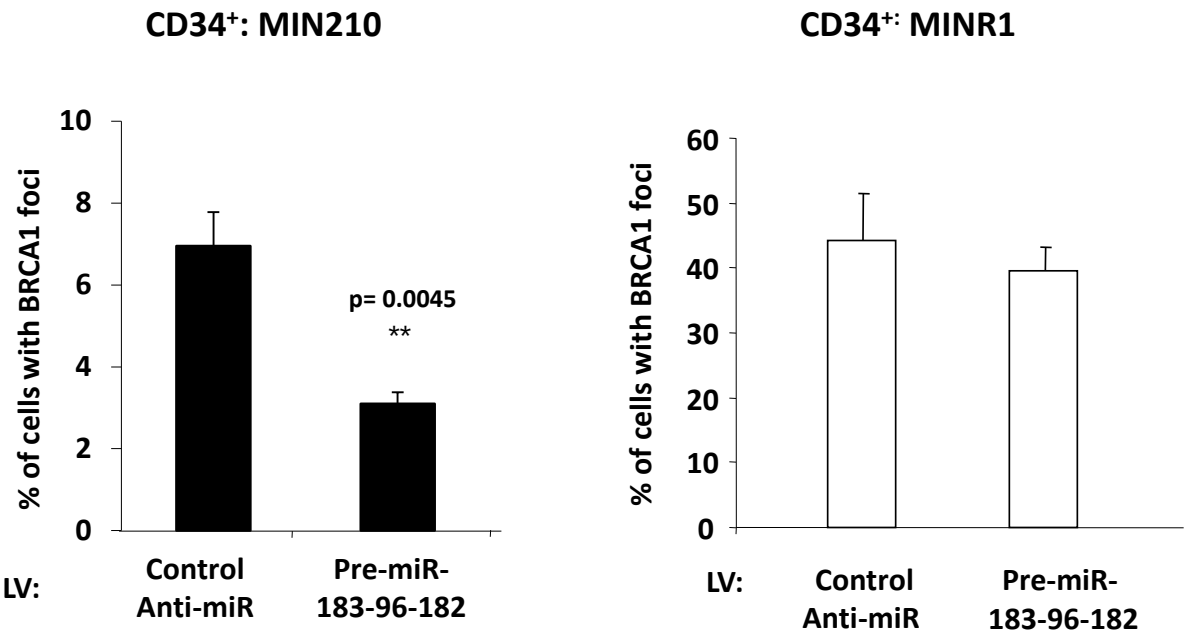
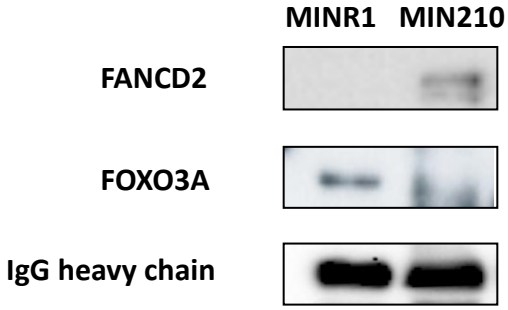

c)
Control Anti-miR
Anti-miR-96
Anti-miR-182
Anti-miR-183
BRCA1
EGFP

Figure 2

d)


CD34+: MIN210
% of cells with BRCA1 foci
10
8
6
4
2
0
p= 0.0045
**
LV:
Control Anti-miR
Pre-miR-183-96-182
CD34+: MINR1
% of cells with BRCA1 foci
60
50
40
30
20
10
0
LV:
Control Anti-miR
Pre-miR-183-96-182

e)


MINR1 MIN210
FANCD2
FOXO3A
IgG heavy chain

f)

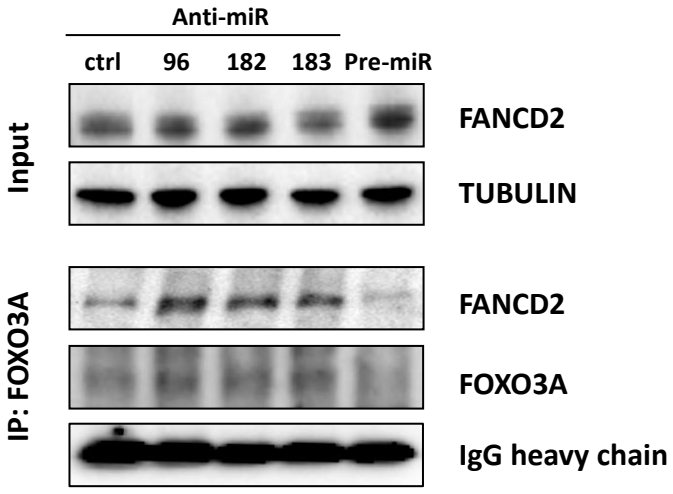
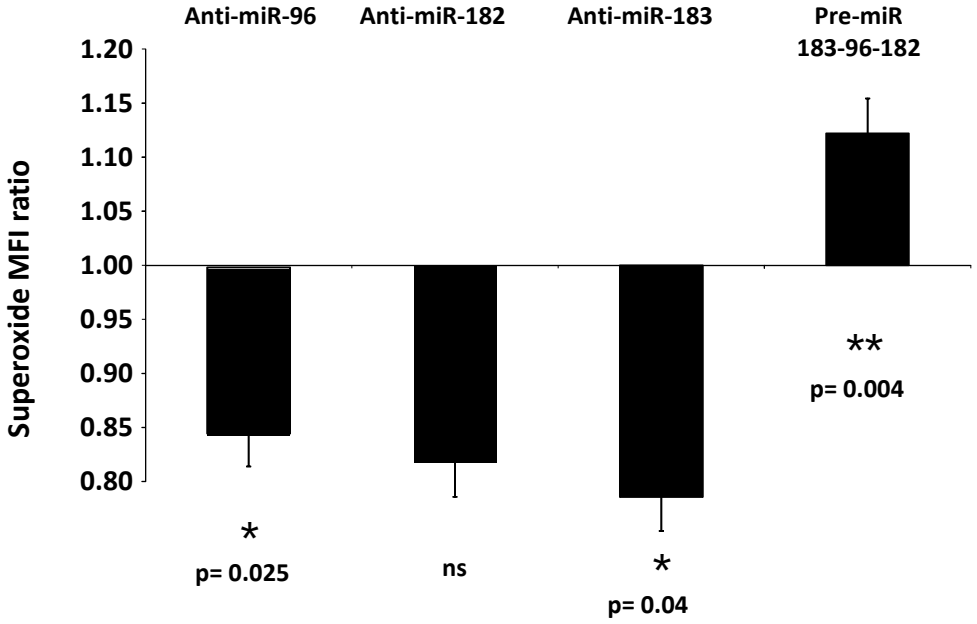
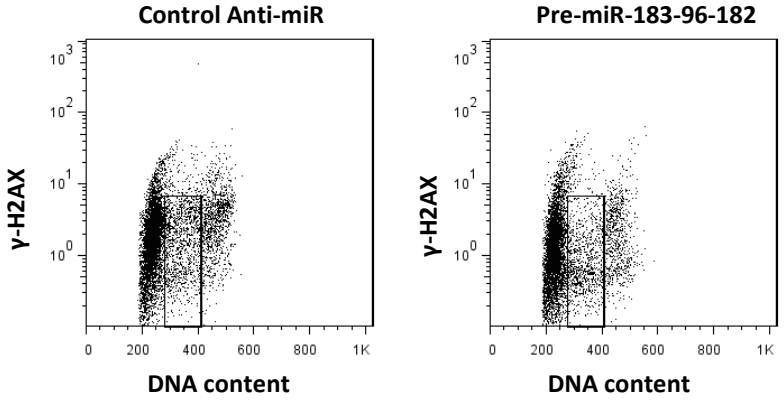

Anti-miR
ctrl 96 182 183 Pre-miR
Input
FANCD2
TUBULIN
IP: FOXO3A
FANCD2
FOXO3A
IgG heavy chain

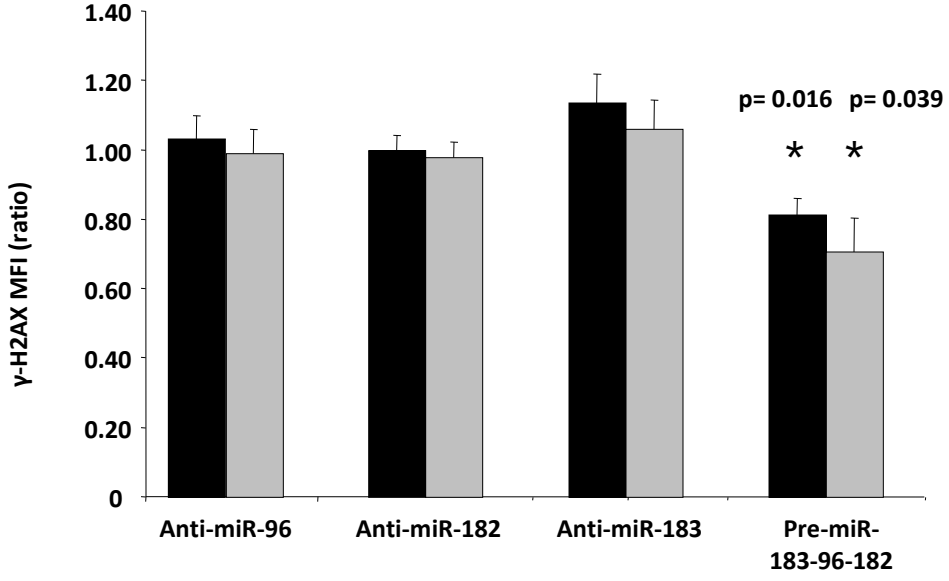
Figure 3

a)

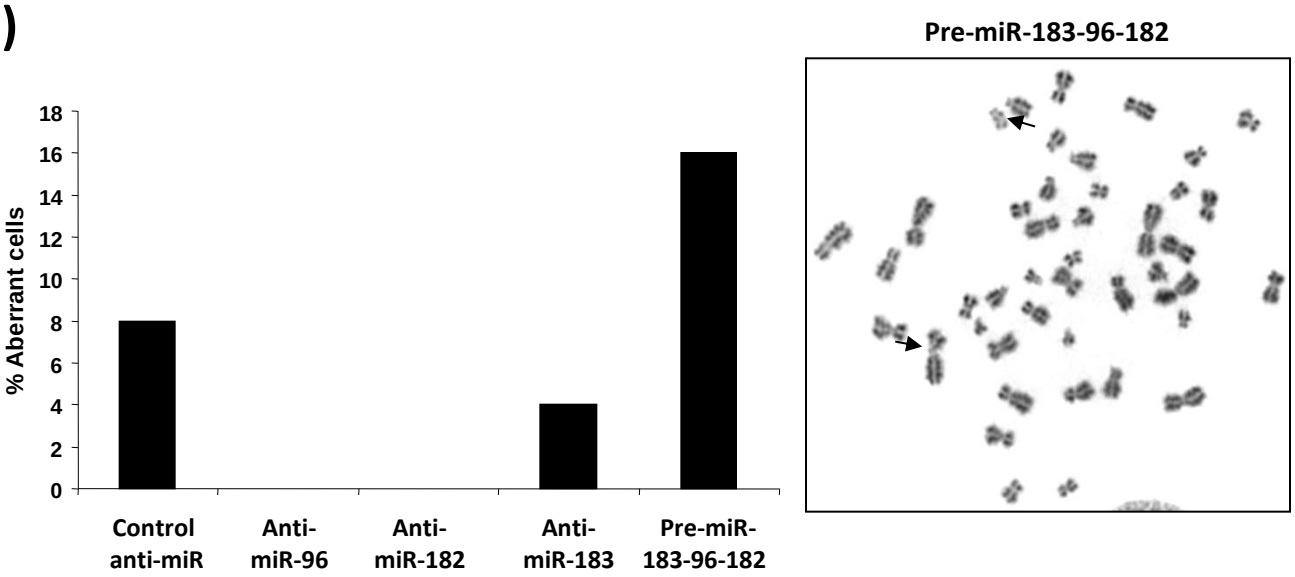

Anti-miR-96
Anti-miR-182
Anti-miR-183
Pre-miR 183-96-182
Superoxide MFI ratio
1.20
1.15
1.10
1.05
1.00
0.95
0.90
0.85
0.80
*
p= 0.025
ns
*
p= 0.04
**
p= 0.004

b)

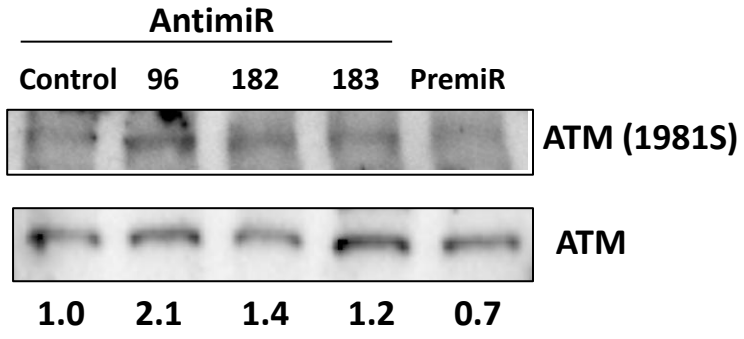

Control Anti-miR
Pre-miR-183-96-182
γ-H2AX
DNA content
0
200
400
600
800
1K


γ-H2AX MFI (ratio)
1.40
1.20
1.00
0.80
0.60
0.40
0.20
0
p= 0.016
p= 0.039
*
*
Anti-miR-96
Anti-miR-182
Anti-miR-183
Pre-miR-183-96-182

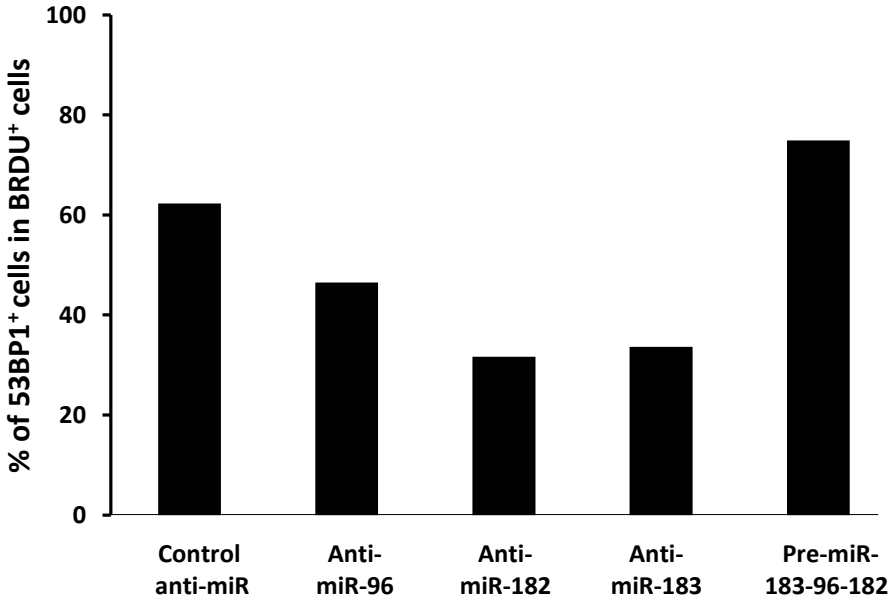
c)


Pre-miR-183-96-182
% Aberrant cells
18
16
14
12
10
8
6
4
2
0
Control anti-miR
Anti-miR-96
Anti-miR-182
Anti-miR-183
Pre-miR-183-96-182

d)


AntimiR
Control
96
182
183
PremiR
ATM (1981S)
ATM
1.0
2.1
1.4
1.2
0.7

e)


100
80
60
40
20
0
% of 53BP1+ cells in BRDU+ cells
Control anti-miR
Anti-miR-96
Anti-miR-182
Anti-miR-183
Pre-miR-183-96-182

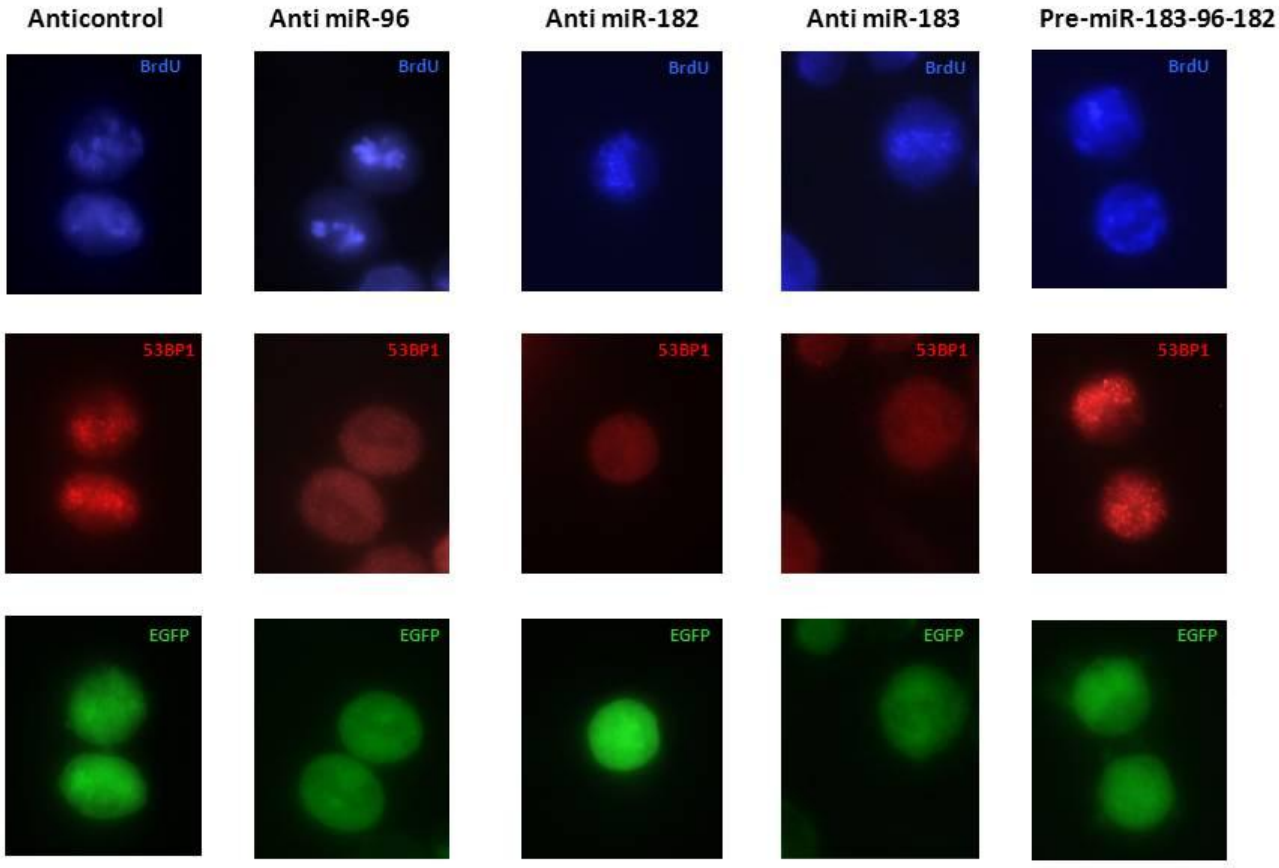
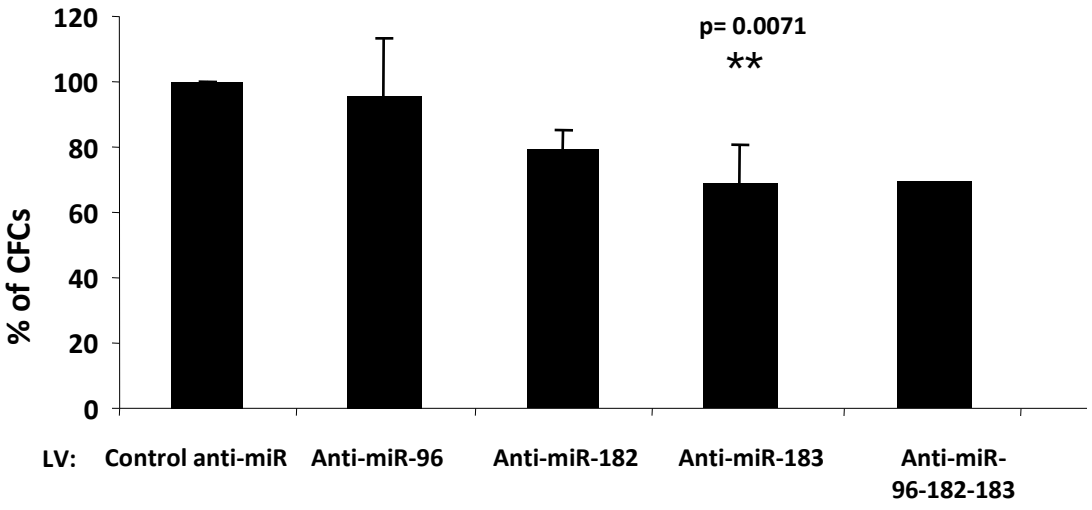
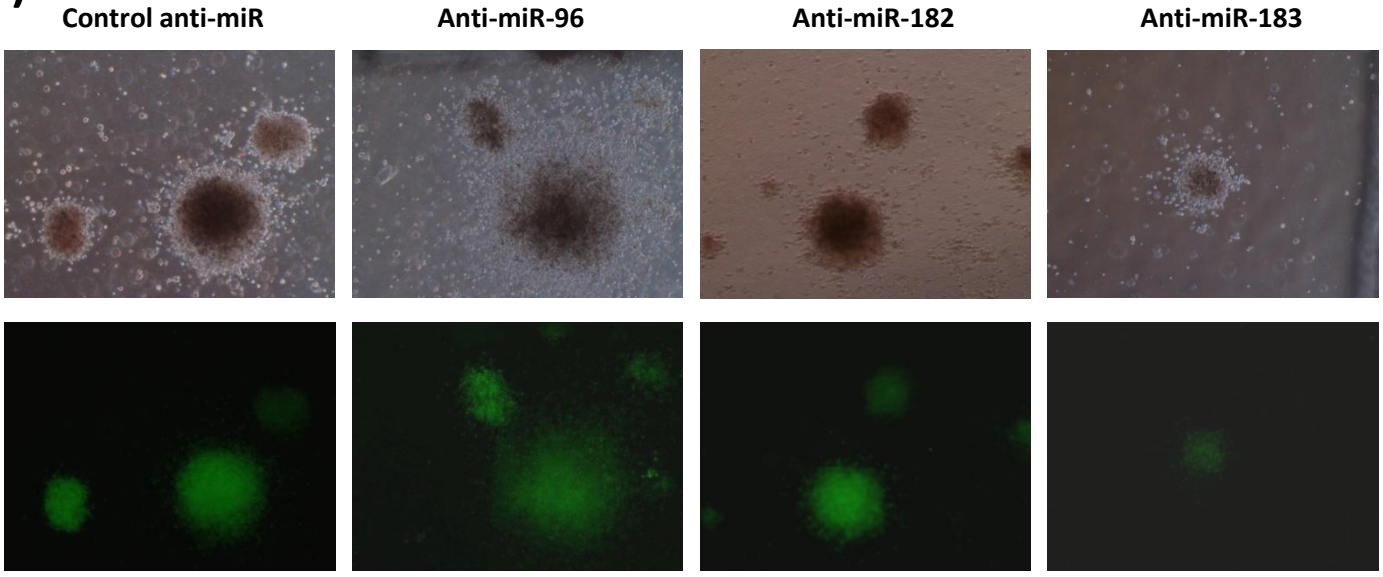

Anticontrol
Anti miR-96
Anti miR-182
Anti miR-183
Pre-miR-183-96-182
BrdU
53BP1
EGFP

Figure 4

a)


CD34+: BCR-ABL1
p= 0.0071
**
% of CFCs
120
100
80
60
40
20
0
LV:
Control anti-miR
Anti-miR-96
Anti-miR-182
Anti-miR-183
Anti-miR-96-182-183

b)


Control anti-miR
Anti-miR-96
Anti-miR-182
Anti-miR-183

c)

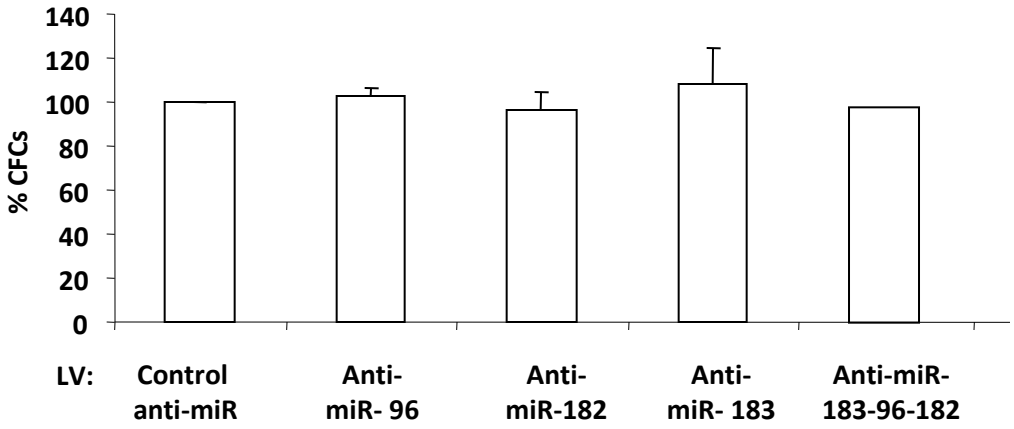
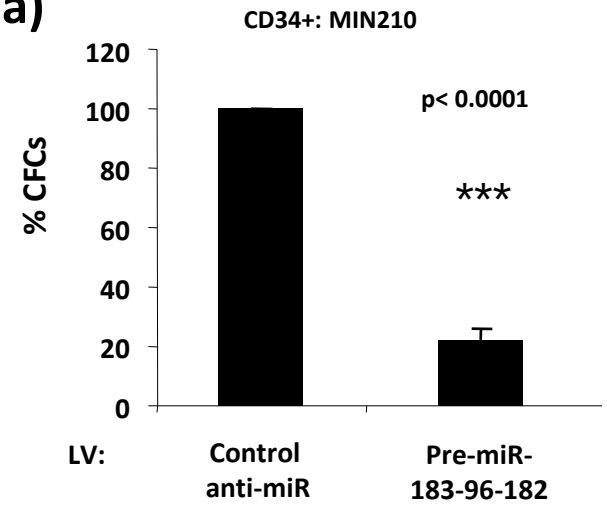
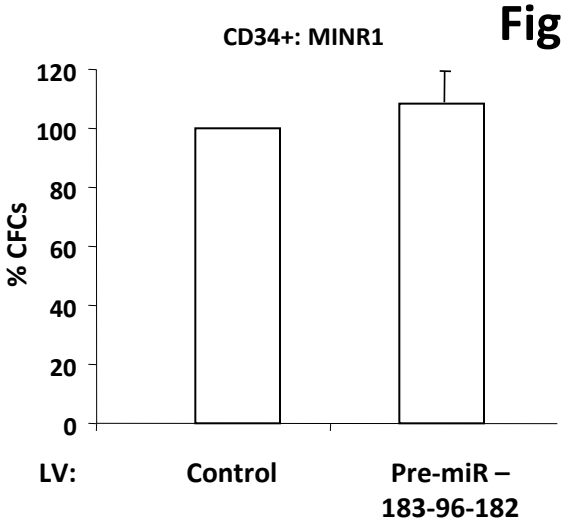

% CFCs
140
120
100
80
60
40
20
0
LV:
Control anti-miR
Anti-miR- 96
Anti-miR-182
Anti-miR- 183
Anti-miR-183-96-182

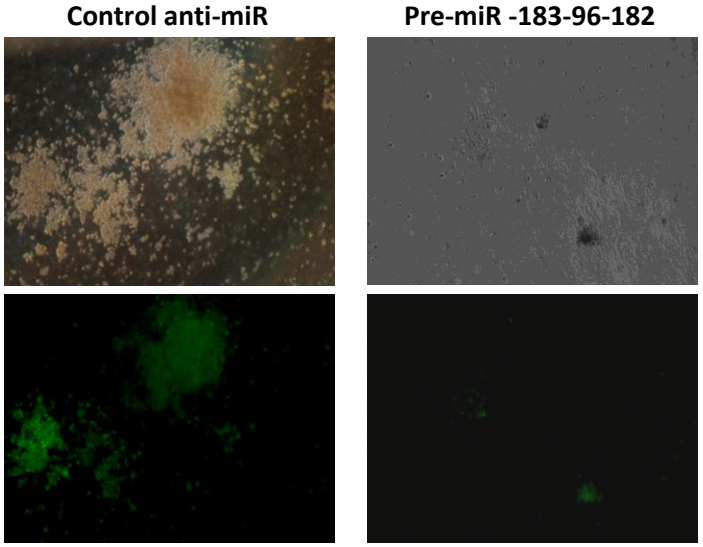
Figure 5

a)

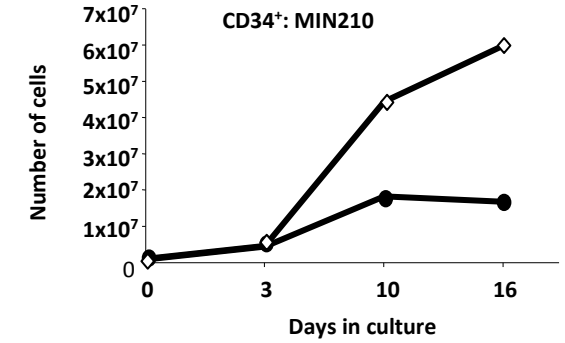


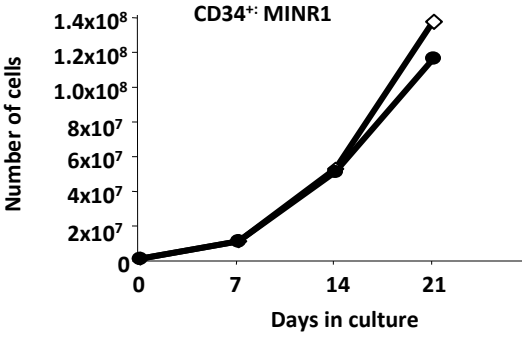


b)

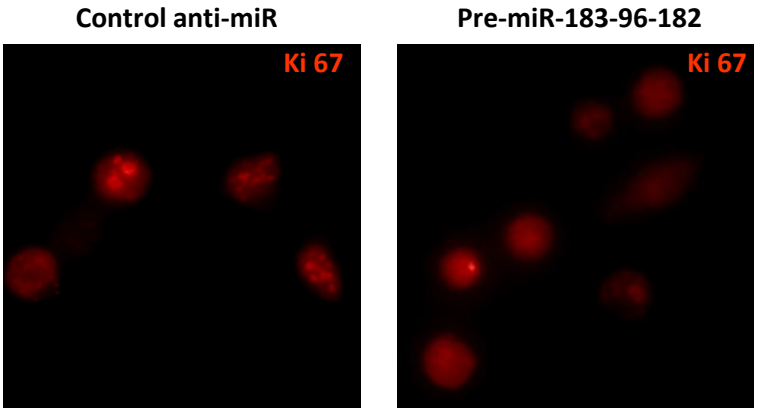


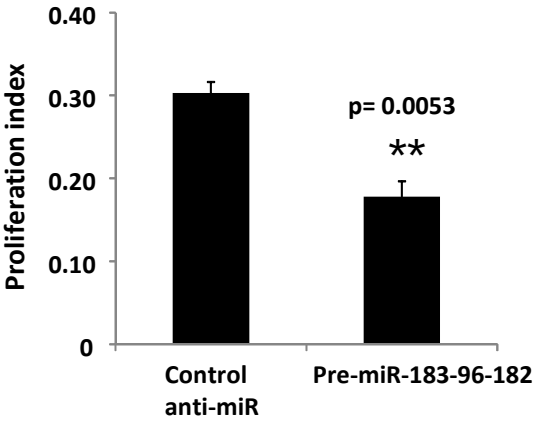
c)





d)





e)

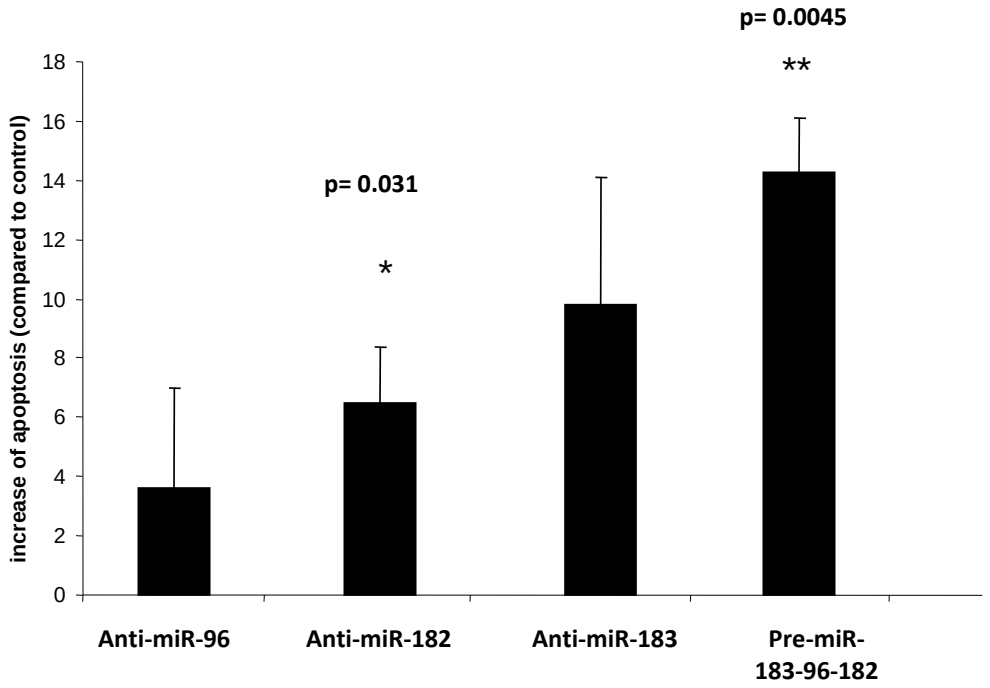
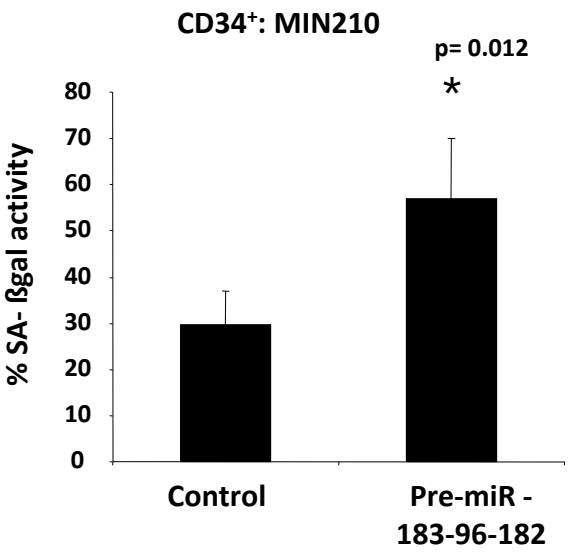
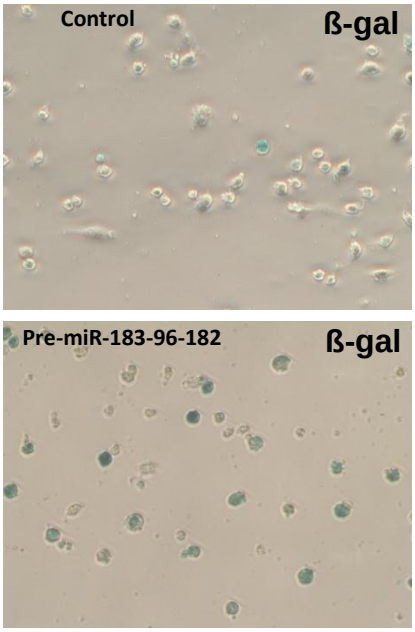

p= 0.0045
**
p= 0.031
*
increase of apoptosis (compared to control)
18
16
14
12
10
8
6
4
2
0
Anti-miR-96
Anti-miR-182
Anti-miR-183
Pre-miR-183-96-182

Figure 6

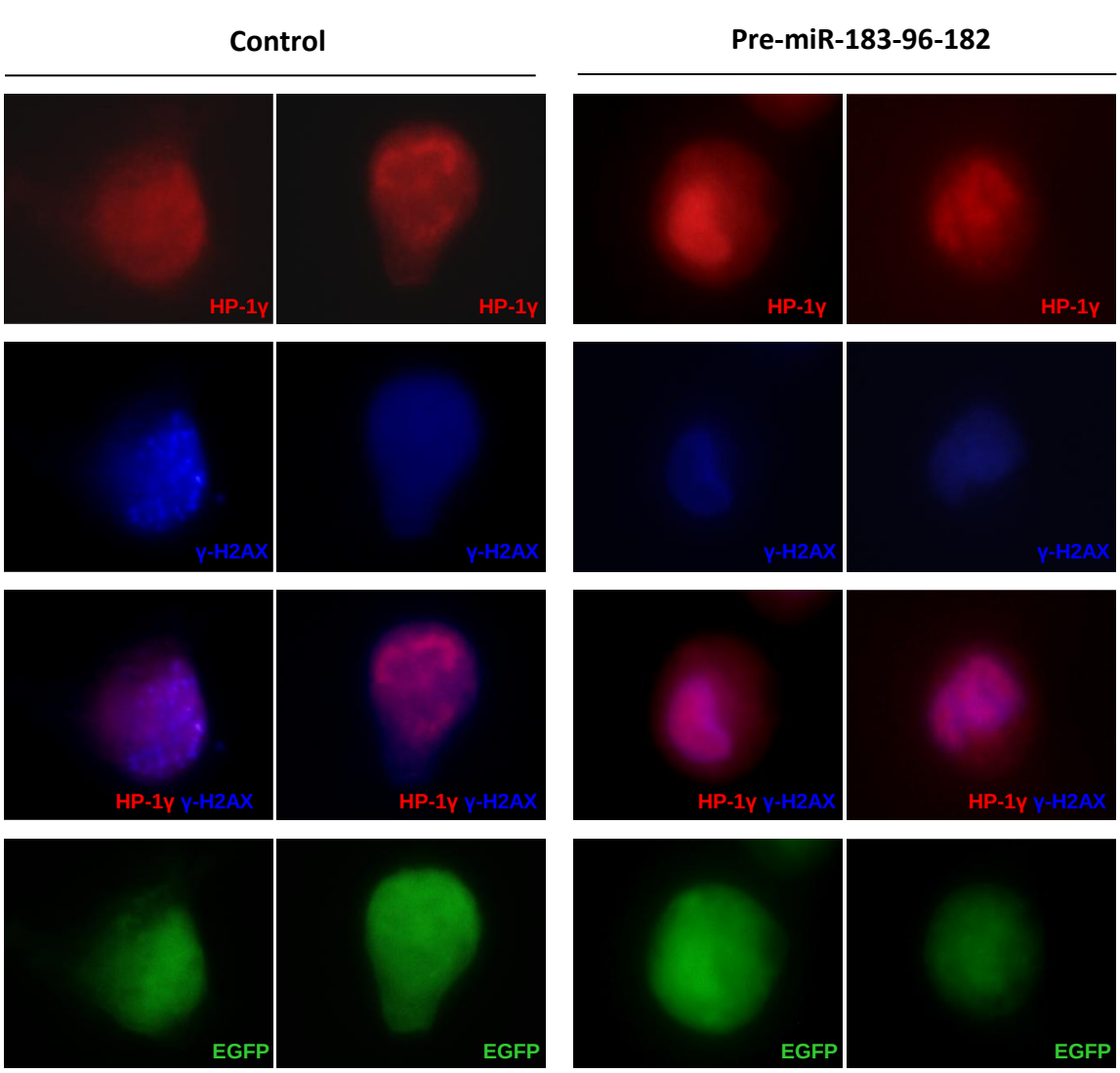
a)


CD34+: MIN210
p= 0.012
*
% SA- ßgal activity
80
70
60
50
40
30
20
10
0
Control
Pre-miR - 183-96-182

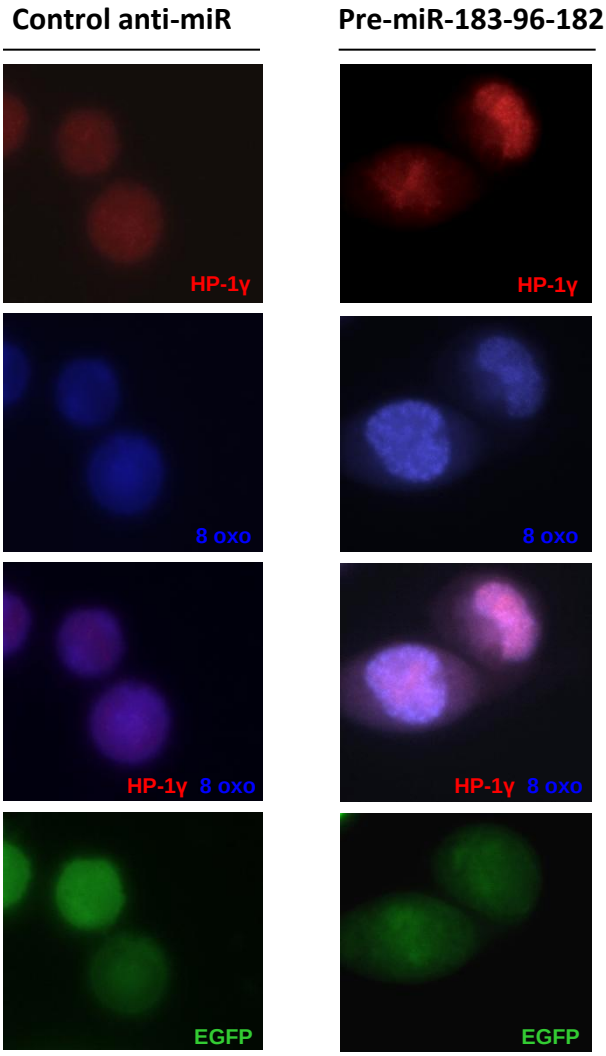
b)


Control
ß-gal
Pre-miR-183-96-182
ß-gal

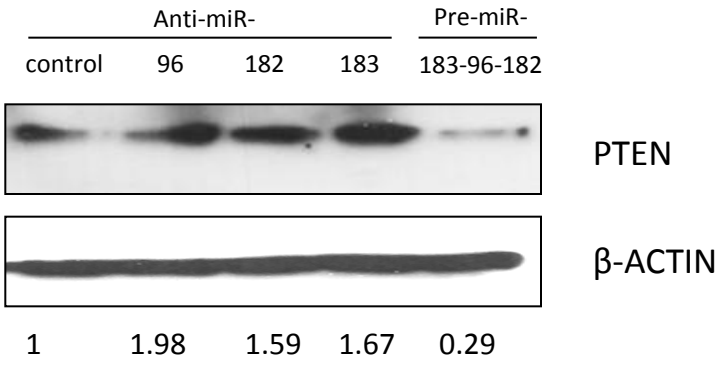
c)


Control
Pre-miR-183-96-182
HP-1γ
γ-H2AX
HP-1γ γ-H2AX
EGFP

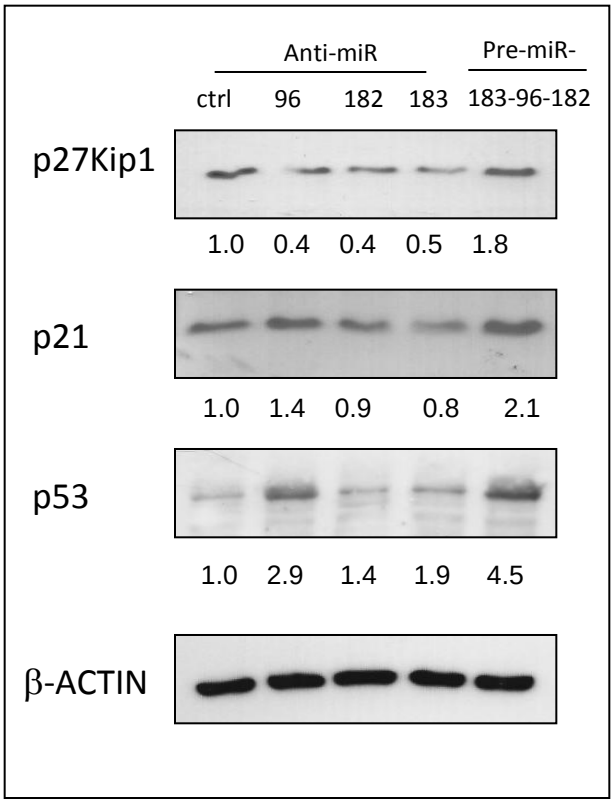
d)

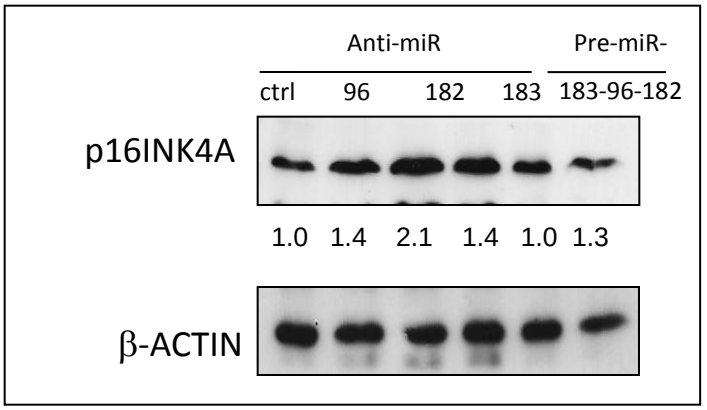

Control anti-miR
Pre-miR-183-96-182
HP-1γ
HP-1γ
8 oxo
8 oxo
HP-1γ 8 oxo
HP-1γ 8 oxo
EGFP
EGFP

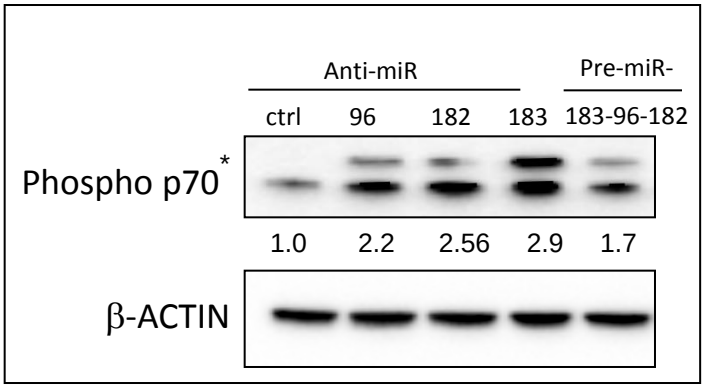
e)

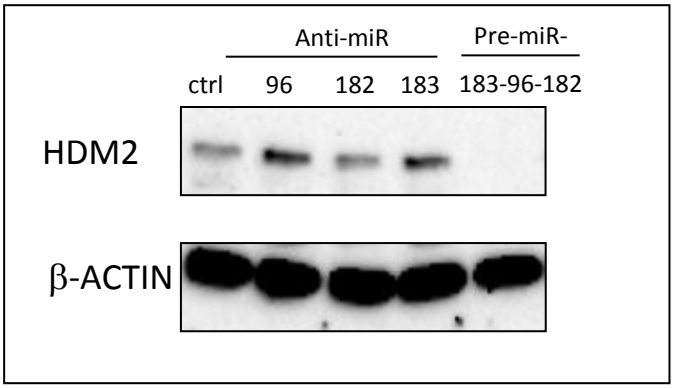

Anti-miR-
Pre-miR-
control
96
182
183
183-96-182
PTEN
β-ACTIN
1
1.98
1.59
1.67
0.29

f)


Anti-miR
Pre-miR-
ctrl
96
182
183
183-96-182
p27Kip1
1.0 0.4 0.4 0.5 1.8
p21
1.0 1.4 0.9 0.8 2.1
p53
1.0 2.9 1.4 1.9 4.5
β-ACTIN

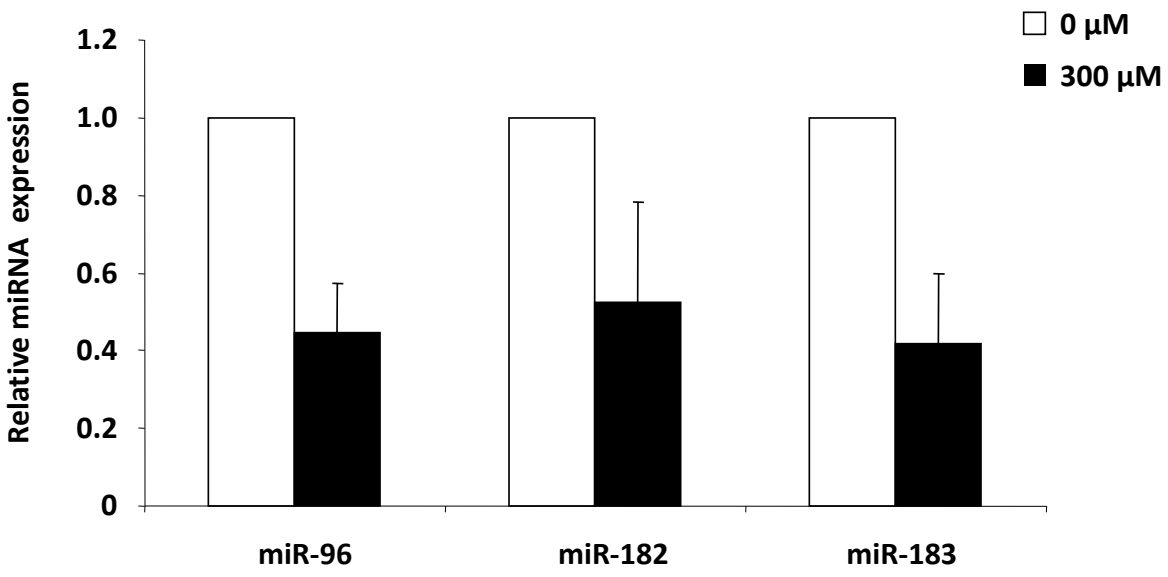

Anti-miR
Pre-miR-
ctrl
96
182
183
183-96-182
p16INK4A
1.0 1.4 2.1 1.4 1.0 1.3
β-ACTIN


Anti-miR
Pre-miR-
ctrl
96
182
183
183-96-182
Phospho p70*
1.0 2.2 2.56 2.9 1.7
β-ACTIN


Anti-miR
Pre-miR-
ctrl
96
182
183
183-96-182
HDM2
β-ACTIN

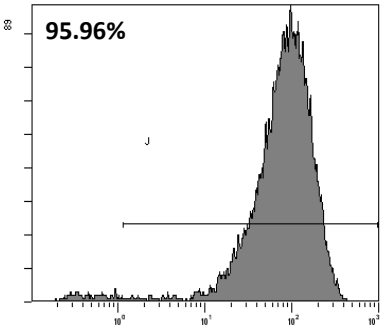
Supplem. Figure 1

a)

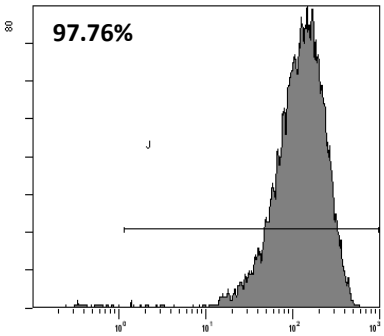


Supplem. Figure 2

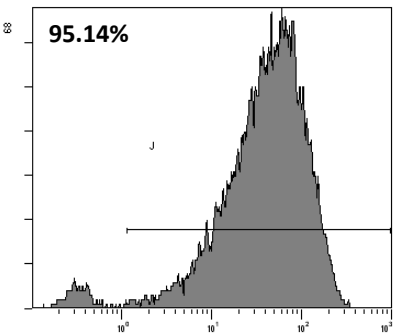
LV: Control Anti-miR



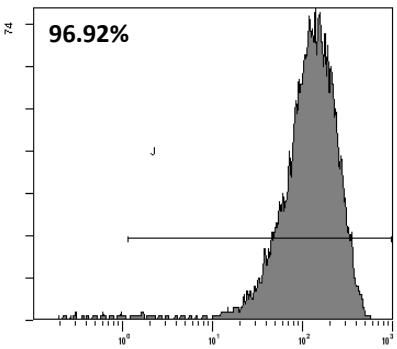
LV :Anti-miR-96



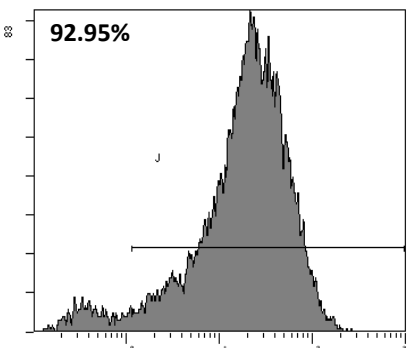
LV: Anti-miR-182



LV: Anti-miR-183

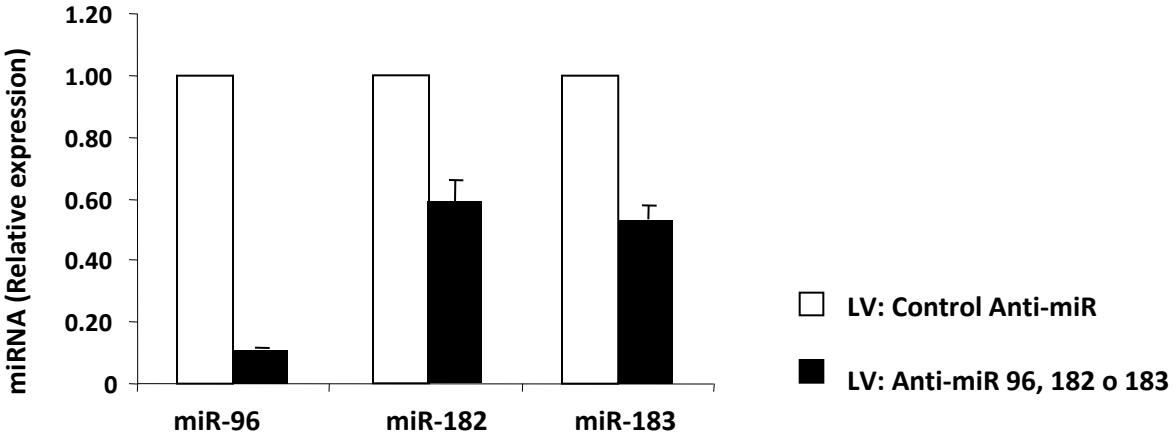


LV: PremiR-183-96-182

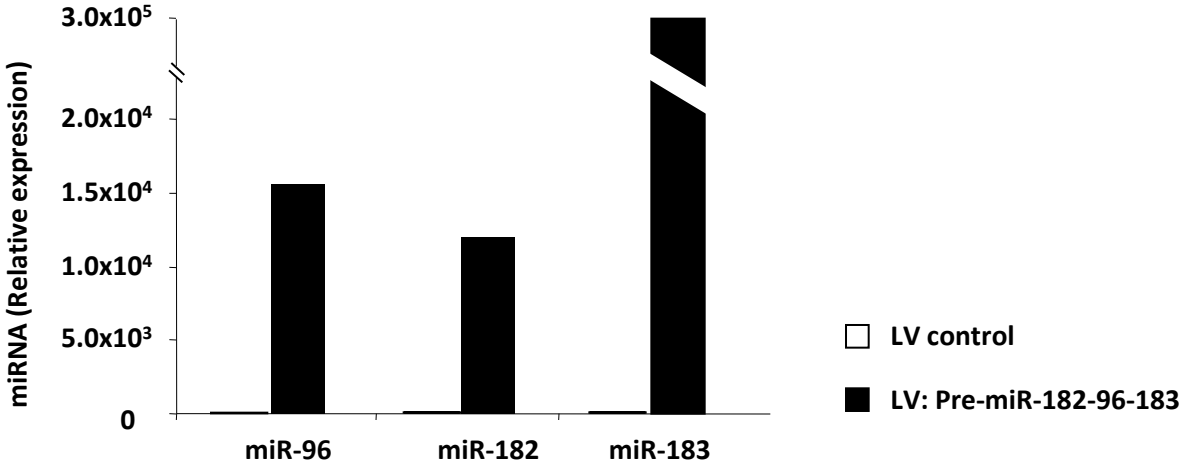


Supplem. Figure 3

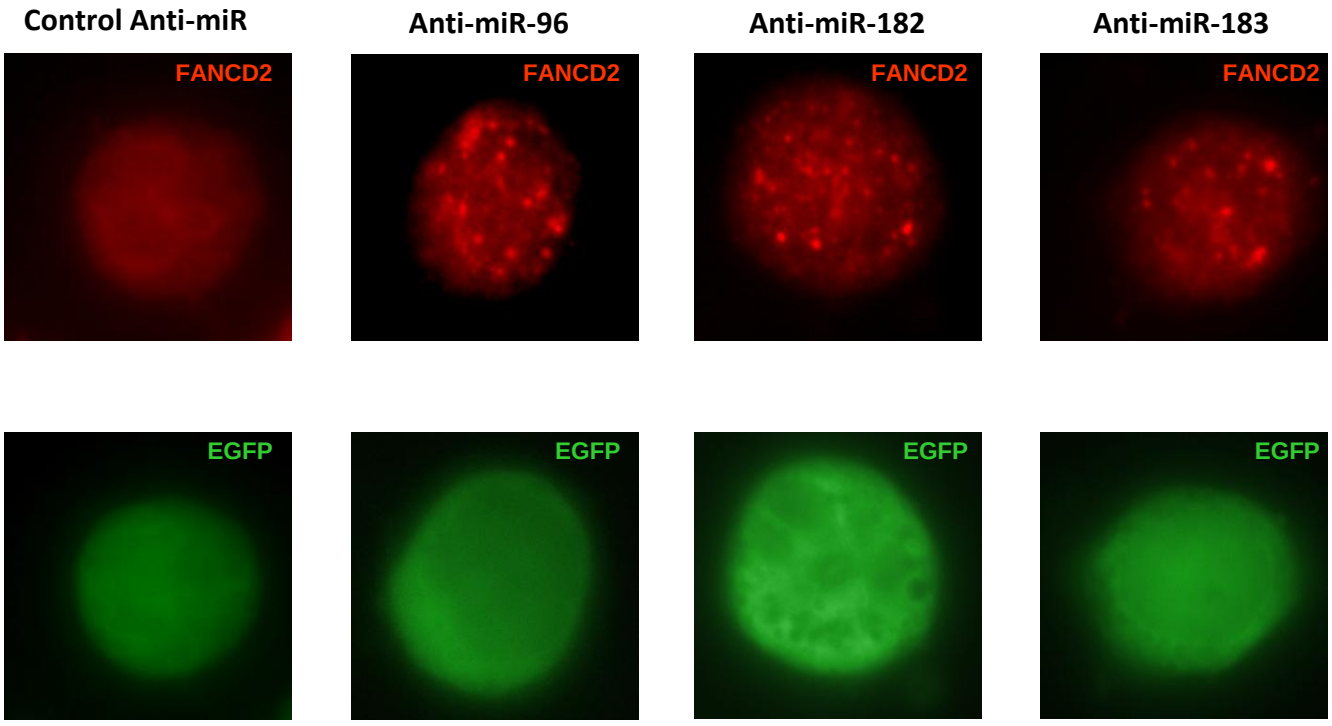
a)



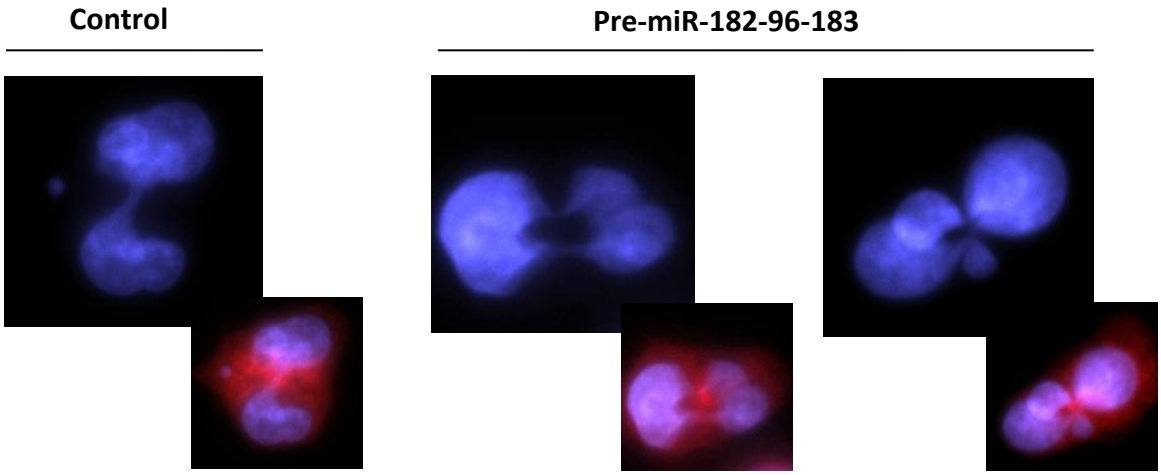
b)

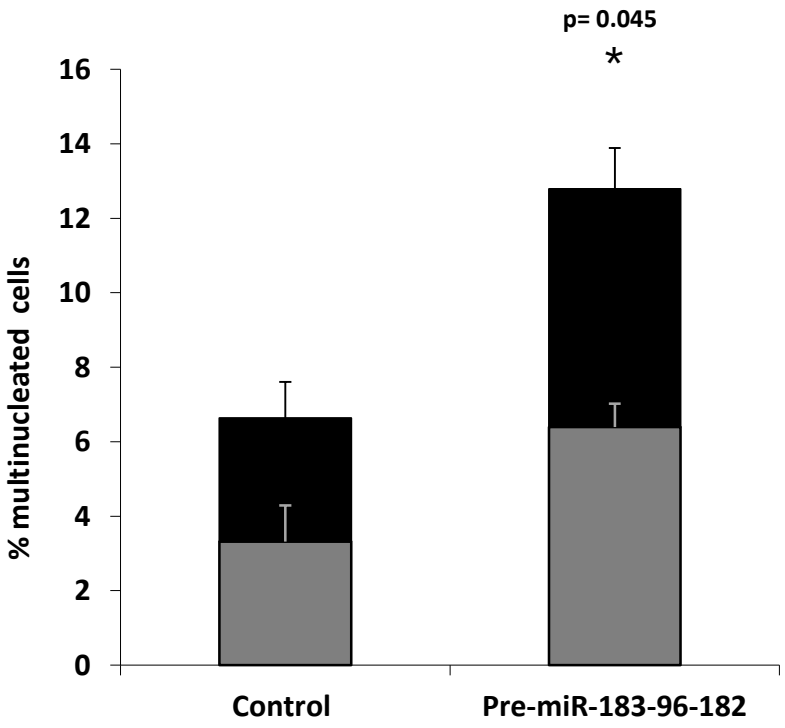


Supplem. Figure 4

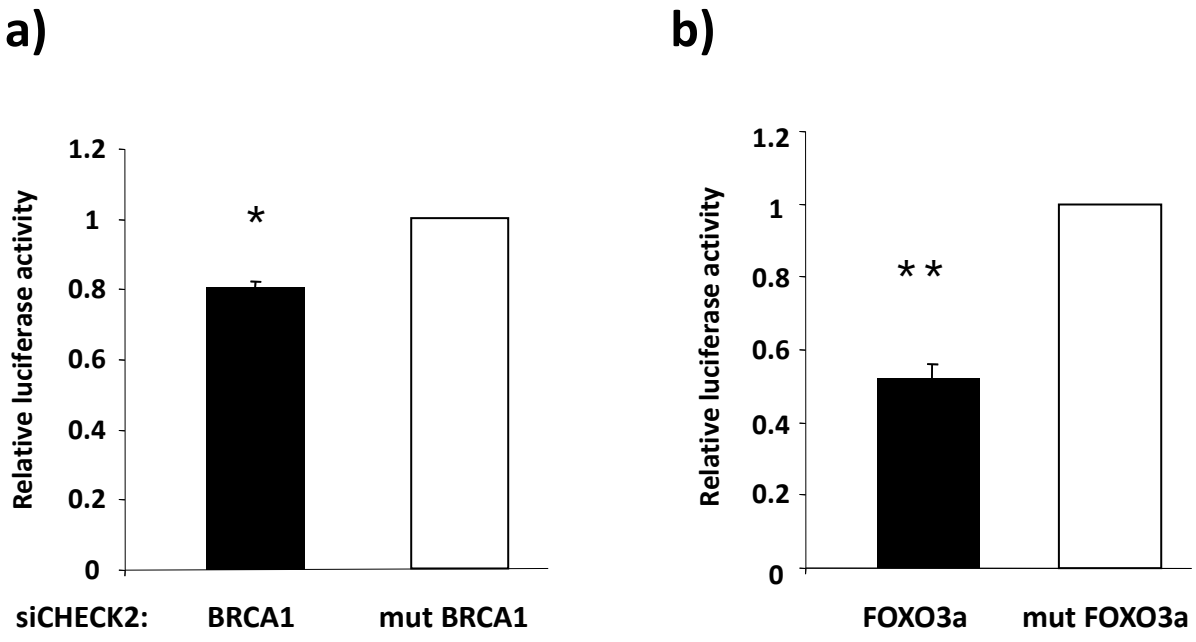


Supplem. Figure 5



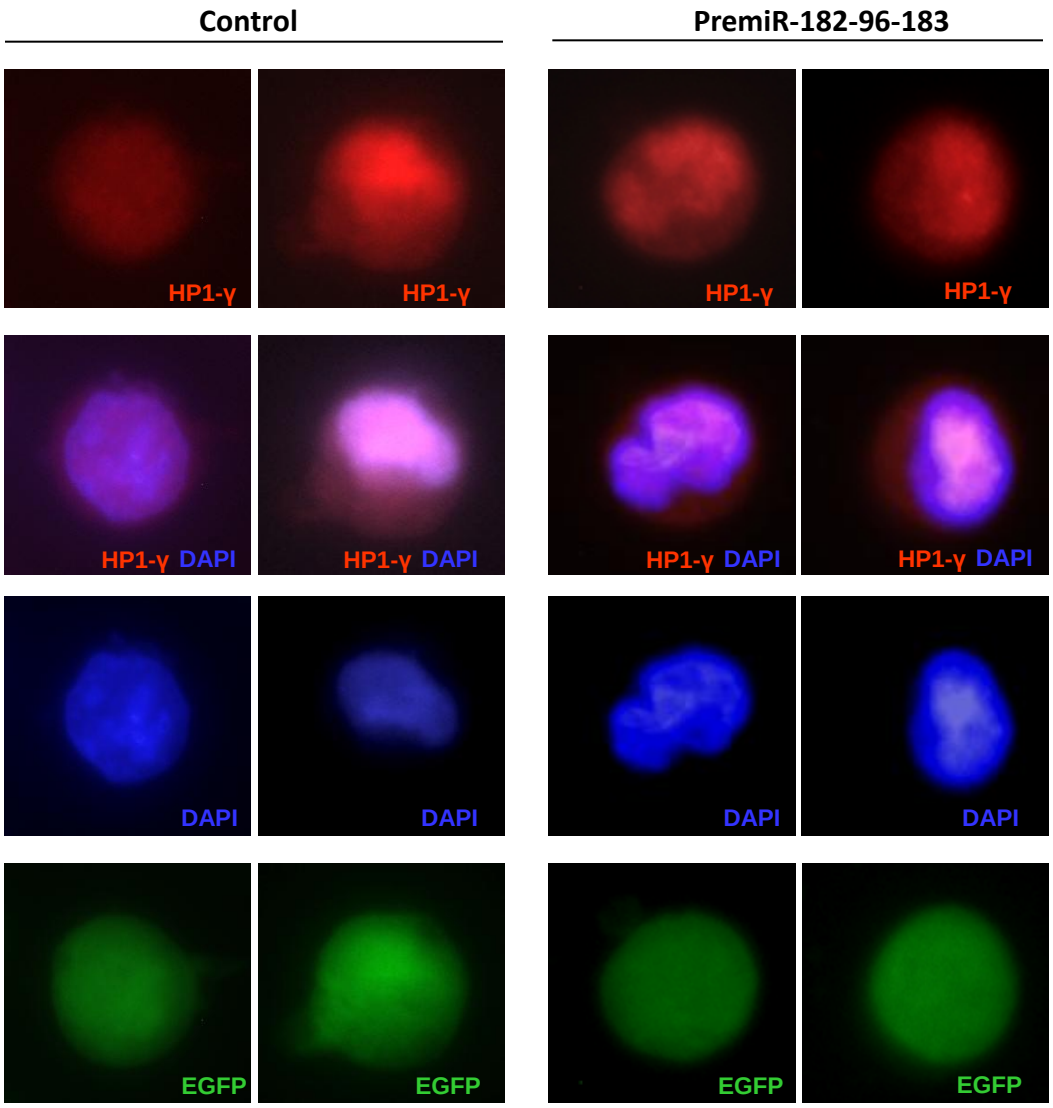


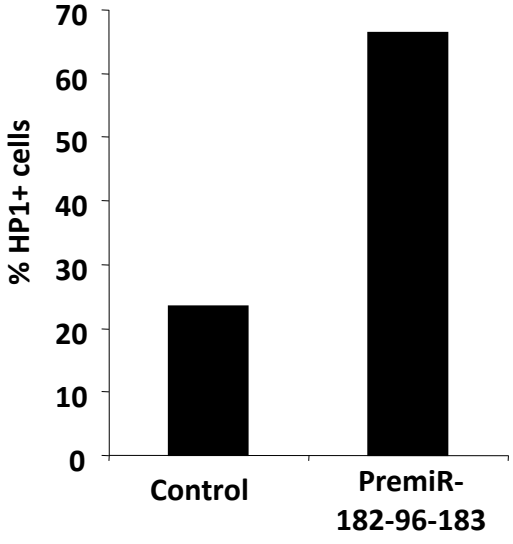
Supplem. Figure 6



Supplem. Figure 7

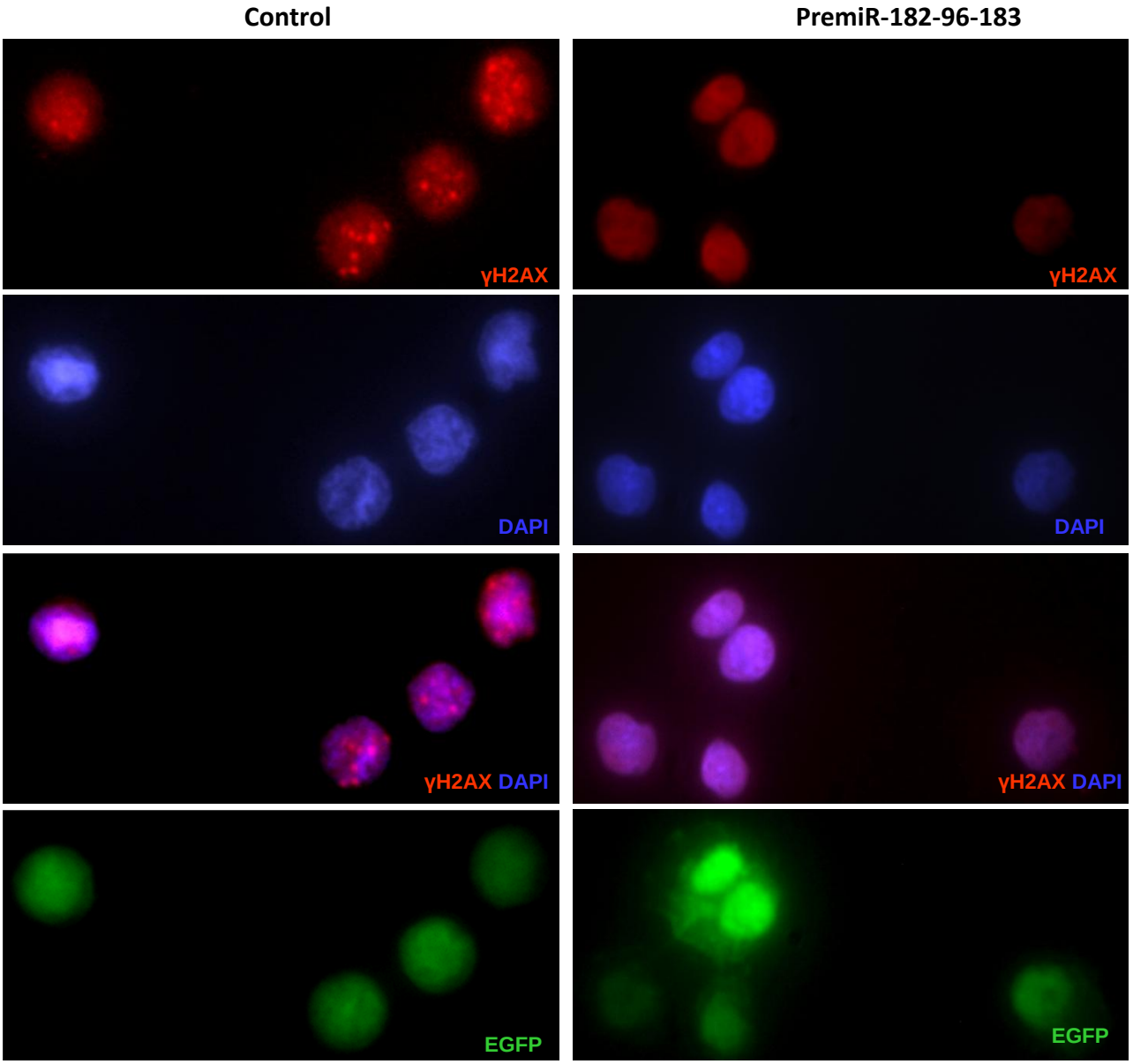
a)



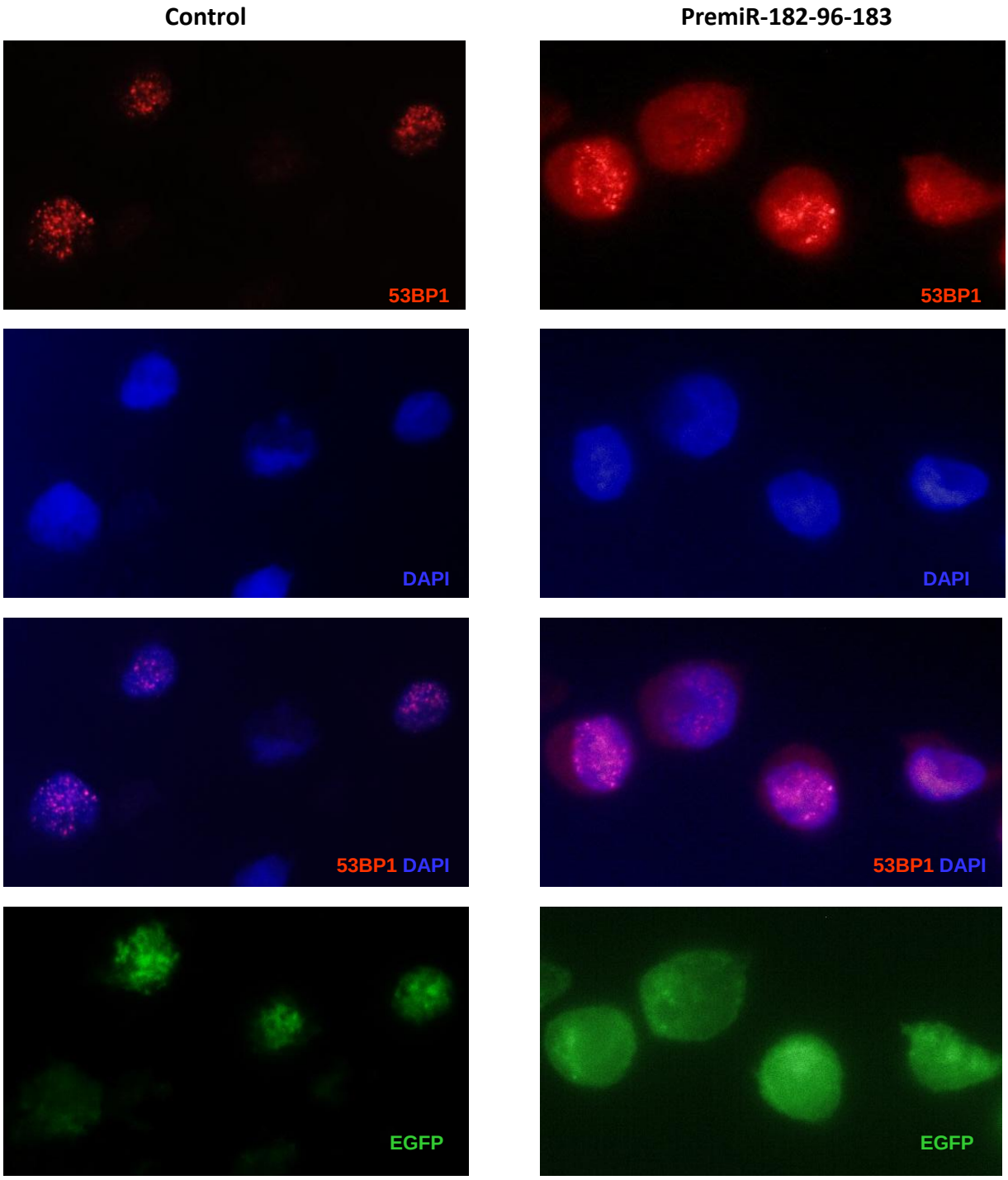


Supplem. Figure 7

b)

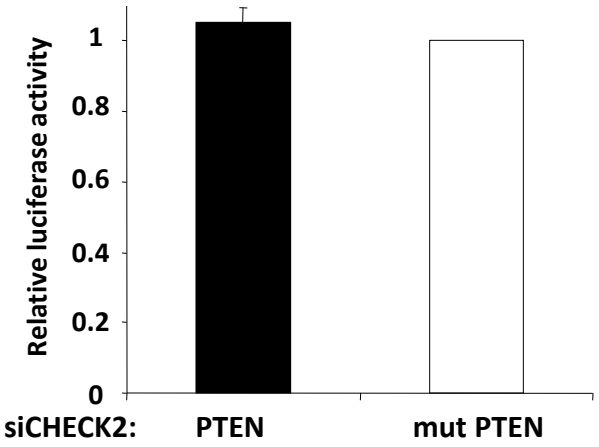


Supplem. Figure 8

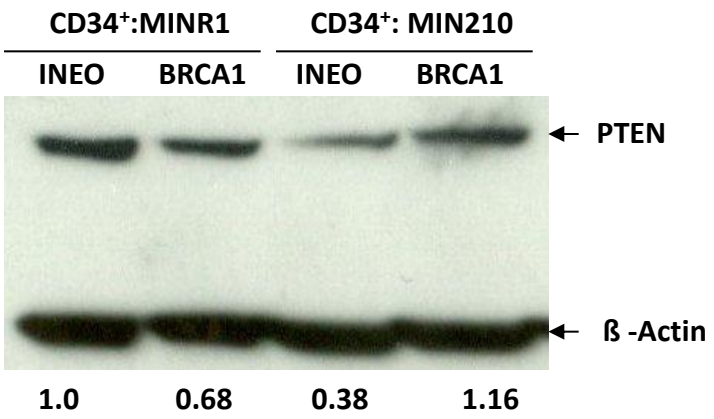


Supplem. Figure 9

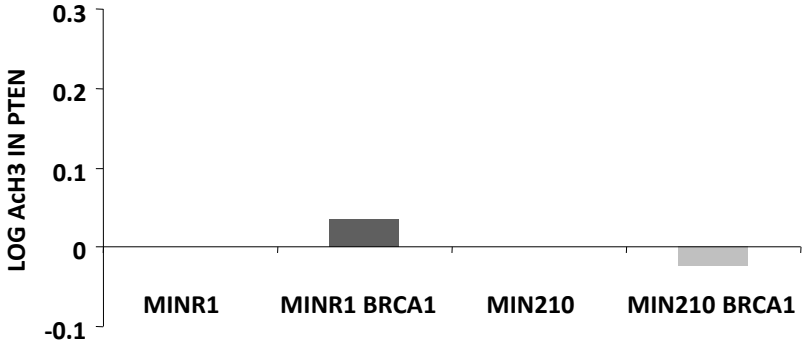
a)



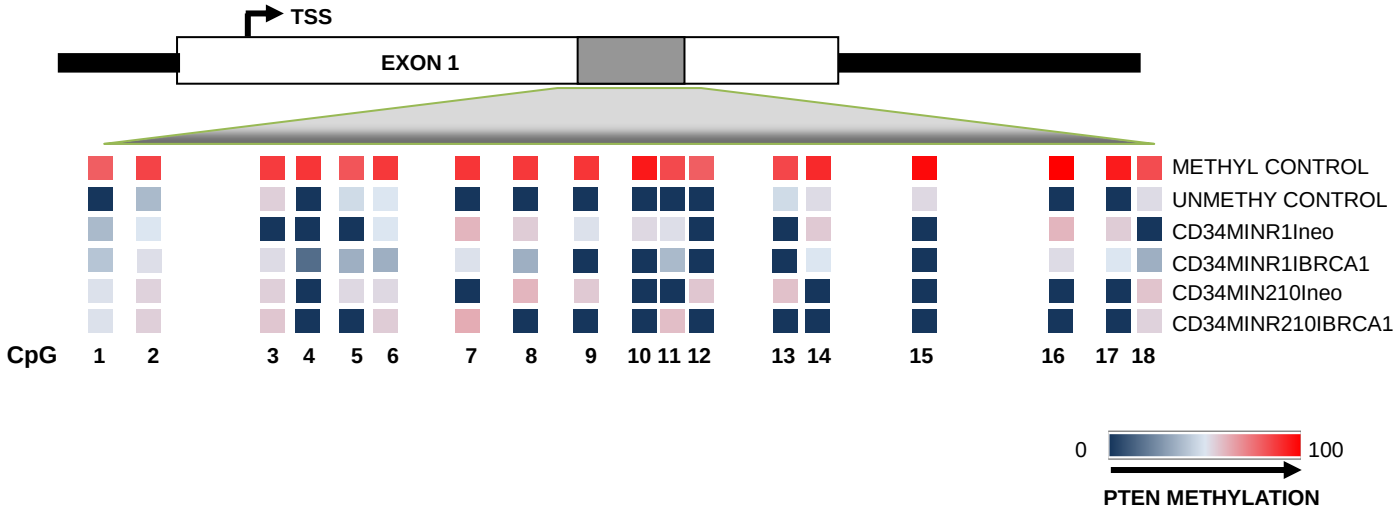
b)



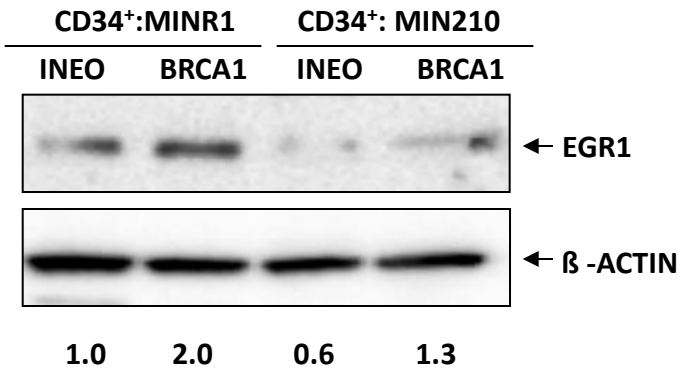
c)



d)


TSS
EXON 1
METHYL CONTROL
UNMETHY CONTROL
CD34MINR1Ineo
CD34MINR1IBRCA1
CD34MIN210Ineo
CD34MINR210IBRCA1
CpG 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
0
100
PTEN METHYLATION

e)


CD34+:MINR1
CD34+: MIN210
INEO
BRCA1
INEO
BRCA1
EGR1
ß -ACTIN
1.0
2.0
0.6
1.3

SUPPLEMENTARY METHODS

Retroviral and lentiviral vectors generation and production.

The control retroviral vector (RV) used in these experiments is the MINR1 retroviral vector which consist on a MSCV-IRES-ΔNGFR vector. The MIN210 is derived from the MINR1, and contains the full-length b3a2 *BCR-ABL1* cDNA under the control of the MSCV promoter. Both vectors were kindly provided by Bryan G. Druker (Oregon Health and Science University, Portland, OR) and the supernatant was obtained as previously described (1).

Lentiviral vectors carrying a control Anti-miR and the different shRNAs against miR-96, miR-182 and miR-183 were constructed using the LV-THM vector, where the EGFP is under the control of the EF-1a promoter and the shRNA is included in the LTR, under the control of the H1 promoter(2). The design of the different shRNAs was based on the mature sequence of the miRNAs and cloned as previously described by Scherr *et al*.(3). A control Anti-miR LV was designed with an shRNA that do not recognize any sequence in the genome. shRNAs for the different microRNAs were cloned in LV-THM vector by annealing of the different primers included in Suplem. Table I. Annealing was conducted incubating both primers 4 min. at 95°C and 10 min. at 70°C in annealing buffer (100mM potassium acetate, 30mM HEPES pH7.4 and 2mM magnesium acetate). Primers were phosphorylated and cloned into the LV-THM vector digested with MluI and ClaI.

To overexpress the miR-182-96-183 cluster a lentiviral vector kindly provided by Segura et al., was used.

Lentiviral infective particles were obtained by transfection of 293T cells with 5µg of pCMVdR8.74, 5µg of VSV-G (both from Plasmid Factory) and 10µg of the different Anti-miR LV or Pre-miR-183-96-182 LV as previously described (4, 5). Forty eight hours after transfection supernatants were collected, filtered through 0.45 µm and concentrated by ultracentrifugation. Functional titers of infective LVs were determined in HT1080 cells as previously described (6) by flow cytometry. Titers around $1x10^8$ TU/ml were obtained.

RNA extraction, reverse transcription and Real-Time PCR quantification of miRNAs.

RNA from $1x10^6$ BM $CD34^+$ cells from H.D. and CML patients or $CD34^+$ cells transduced either with MINR1 or MIN210 was extracted using Trizol total RNA isolation

reagent (Invitrogen). cDNA was synthesized using gene specific primers for miR-96, mir-182, miR-183 and *RNU6B* according to the Taqman MicroRNA Assay protocol (PE Applied Biosystems, Foster City, CA). Reaction was prepared as previously published (7). Real Time qPCR was performed using a Rotor Gene system. The reaction was incubated at 95ºC for 10 min, followed by 40 cycles of 95ºC for 15s and 60ºC for 10 min. The Ct data was calculated using default threshold settings. Normalization of the data was done using *RNU6B* gene as endogenous control. Relative quantification of expression of analyzed miRNAs was calculated with the $2^{-\Delta\Delta Ct}$ method (Applied Biosystems. User Bulletin N°2 (P/N 4303859)). Data are presented as log $2^{-\Delta\Delta Ct}$ of the relative quantity of miRNAs, normalized and compared with the relative medium expression value of $CD34^{+}$: MINR1.

Luciferase reporter assays.

For reporter assays siCHECK2 vector (Promega) was used. A region of WT 3´UTR from *BRCA1*, the 3'UTR from *BRCA1* mutated, 3'UTR from *FOXO3A,* its mutated version and the 3'UTR from PTEN or its mutated version were constructed annealing the primers included in Supplementary table II.

Annealing was conducted incubating both primers 4 min. at 95°C and 10 min. at 70°C in annealing buffer. Primers were phosphorylated and cloned into the siCHECK2 vector from Promega digested with Xho I and Not I.

RKO cells were stably transduced with the LV: control or a LV: PremiR96-182-183 to reach more than 95% of transduced cells and transfected with the siCHECK2 vectors with the different regions of the 3'UTR.

24h post-transfection luciferase reporter assay was conducted using Dual-Glo luciferase kit (Promega). The ratio between the *Firefly* and the *Renilla* luciferase allows the normalization of luciferase values. Ratios were normalized against the control plasmid. Experiments were conducted in six replicates in at least three different experiments. In all cases the overexpression of the different microRNAs was analyzed by RT-qPCR.

DNA methylation analysis of *PTEN.*

We analysed de DNA methylation level of 18 CpG dinucleotides by pyrosequencing. After bisulfite treatment of DNA, PCR using a Pyromark PCR kit (Qiagen, Hilden, Gernamy) and primers PTEN-PYR-1 (5´-GGTTGGTATATTTAGGGATT-3´) and PTEN-PYR-2 (5´-BIOTIN-CTAAACCTACTTCTCCTCAACAACC-3´), was performed with a denaturalization at 95ºC for 15 min and 45 cycles (consisting of denaturation at 94ºC for 1 min, annealing of universal-biotynilated primers at 54ºC for 1 min, and extension

at 72ºC for 1 min) followed by a final 10 min extension. The resulting biotinylated PCR product was immobilized to streptavidin Sepharose High Performance beads (GE healthcare, Uppsala, Sweden) and processed to yield high quality ssDNA using the PyroMark Vacuum Prep Workstation (Biotage, Uppsala, Sweden), according to the manufacturer´s instructions. The pyrosequencing reaction was performed using the PTEN-PYR-1 primer in the Pyromark^{TM} ID (Biotage, Uppsala, Sweden) and sequence analysis was performed using the PyroQ-CpG analysis software (Biotage, Uppsala, Sweden). Water blanks were included with each assay.

Quantitative Chromatin immunoprecipitation (Q-PCR-ChIP) of *PTEN.*

Q-PCR-ChIP assay were performed to assess the level of acetylation of Histone 3 (AcH3: 06-599, Millipore). Chromatin immunoprecipitation (ChIP) was performed as previously described (8). Immunoprecipitated DNA fractions (5ng) from antibody-bound and input chromatin were analyzed in triplicate by quantitative real-time PCR using SYBR Green detection. The amplification of the immunoprecipitation fraction was used as a target and input DNA as reference sequence. *Q*-PCR-ChIP amplifications were carried out with 40 cycles at 95ºC for 15 s and 60ºC for 1 min using the primers PTEN-CHIP-1 (5´-TCACCTGGTCCTTTTCACCT-3´) and PTEN-CHIP-2 (5´-GGGTGGAGGACTGATGATGA-3´).

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Supplementary Tables

Table I: Primers used for the desing of anti-miR:LV.

Name	Primer sequence 5'-->3'
Anti-miR control. F	CGCGTCCCAAATCCTTTAGACCGAGCGTGTGTTTTTTGGAAAT
Anti-miR control. R	CGATTTCCAAAAAACACACGCTCGGTCTAAAGGATTTGGGA
Anti-miR-96. F	CGCGTCCCAGCAAAAATGTGCTAGTGCCAAATTTTTTGGAAAT
Anti-miR-96. R	CGATTTCCAAAAAATTTGGCACTAGCACATTTTTGCTGGGA
Anti-miR-182. F	CGCGTCCCAGTGTGAGTTCTACCATTGCCAAATTTTTTGGAAAT
Anti-miR-182. R	CGATTTCCAAAAAATTTGGCAATGGTAGAACTCACACTGGGA
Anti-miR-183. F	CGCGTCCCAGTGAATTCTACCAGTGCCATATTTTTTGGAAAT
Anti-miR-183. R	CGATTTCCAAAAAATATGGCACTGGTAGAATTCACTGGGA

Table II. Primers designed for the cloning of a wild type and mutant 3'UTR region of BRCA1, FOXO3a and PTEN in siCHECK2 vector.

Name	Primer sequence 5'-->3'
BRCA1.Forward	TCGAGGTGTTGGACAGTGTAGCACTCTACCAGTGCCAGGAGCTGGACACCGC
BRCA1.Reverse	GGCCGCGGTGTCCAGCTCCTGGCACTGGTAGAGTGCTACACTGTCCAACACC
mut BRCA1.Forward	TCGAGGTGTTGGACAGTGTAGCACTCTACGCAATGAAGGAGCTGGACACCGC
mut BRCA1.Reverse	GGCCGCGGTGTCCAGCTCCTTCATTGCGTAGAGTGCTACACTGTCCAACACC
FOXO3a.Forward	TCGAGAAAGAAATCAGCTCAGCTCTCCACTCATTGCCAAATGTCACTAAAGGC
FOXO3a.Reverse	GGCCGCCTTTAGTGACATTTGGCAATGAGTGGAGAGCTGAGCTGATTTCTTTC
mut FOXO3a.Forward	TCGAGAAAGAAATCAGCTCCACTTGCCACTCCGGATACGATGTCACTAAAGGC
mut FOXO3a.Reverse	GGCCGCCTTTAGTGACATCGTATCCGGAGTGGCAAGTGGAGCTGATTTCTTTC
PTEN.Forward	TCGAGCCTCTTGAACATGTTTGCCATACTTTTAAAAGGGTAGTTGAAGC
PTEN.Reverse	GGCCGCTTCAACTACCCTTTTAAAAGTATGGCAAACATGTTCAAGAGGC
mut PTEN.Forward	TCGAGCCTCTTGAACATGTTACGTCCACTTTTAAAAGGGTAGTTGAAGC
mut PTEN.Reverse	GGCCGCTTCAACTACCCTTTTAAAAGTGGACGTAACATGTTCAAGAGGC

VIII. DISCUSIÓN

Este estudio tiene como objetivo aportar nueva información que facilite la comprensión de los mecanismos moleculares que participan en la inestabilidad genética de la LMC. En concreto, nuestra hipótesis de partida se centra en que alteraciones en la ruta de la AF/BRCA podrían tener un papel importante en dicha inestabilidad. Esta hipótesis está basada en dos evidencias previas. La primera se debe a la relevancia de la ruta de la AF/BRCA en el mantenimiento de la estabilidad genética de la célula (Levitus et al., 2006; Moldovan and D'Andrea, 2009) y la segunda, en la observación de alteraciones genéticas y epigenéticas en genes de la ruta AF/BRCA o en los componentes que la regulan, en diversos cánceres hereditarios y adquiridos (Hess et al., 2008; Lyakhovich and Surralles, 2006; Narayan et al., 2004; Taniguchi et al., 2003; van der Heijden et al., 2004).

1. Funcionamiento de la ruta de la AF/BRCA en los progenitores hematopoyéticos de pacientes con LMC.

Los experimentos con los que se inició el trabajo estaban encaminados a investigar la capacidad de las células de LMC para generar *foci* nucleares de reparación de FANCD2, que constituye un proceso central en la ruta de la AF (Garcia-Higuera et al., 2001), en condiciones de proliferación celular y tras el daño con agentes entrecruzantes del ADN. Las primeras observaciones en células Mo7e-P210 y células $CD34^+$ de pacientes con LMC indicaron que, al contrario que sus controles sanos, una proporción muy baja de las células que portan el oncogén *BCR-ABL1* generaban *foci* de FANCD2, incluso después del tratamiento con MMC (Artículo 1; figura 1). Como las células de la línea celular Mo7e-p210 y las células primarias de LMC acumulan mutaciones secundarias a la generación de la translocación *BCR-ABL1*, que pueden ser responsables de la alteración en la formación de *foci* de FANCD2, en los experimentos siguientes se utilizaron como modelo células progenitoras $CD34^+$ de SCU, transducidas con vectores retrovirales que expresan el oncogén *BCR-ABL1*. Aunque la expresión ectópica del oncogén produce niveles superiores de la proteína BCR-ABL1 a los detectados en células primarias de pacientes con LMC, estudios previos demostraron que las células humanas transducidas con vectores *BCR-ABL1* reproducen muchas de las características que se observan en progenitores de LMC, facilitando el estudio de los mecanismos moleculares responsables de la transformación en la enfermedad (Chalandon et al., 2002; Zhao et al., 2001).

Los resultados utilizando células $CD34^+$ de SCU demostraron que la mera expresión de BCR-ABL1 mediante la transducción con vectores retrovirales es suficiente para

inhibir la formación de *foci* de FANCD2 en las células BCR-ABL1. Además, imatinib fue capaz de restaurar la generación de *foci* de FANCD2 en las células BCR-ABL1 (Artículo 1; figura 2) poniéndose de manifiesto la relevancia de la actividad cinasa de BCR-ABL1 para inhibir la formación de estos *foci*.

Como para que se produzca la acumulación de *foci* nucleares de FANCD2 se requiere la monoubiquitinación de la proteína (Garcia-Higuera et al., 2001), investigamos esta modificación en la proteína FANCD2 extraída de las células $CD34^+$ BCR-ABL1. Los resultados mostraron una monoubiquitinación eficiente tanto en células expuestas como no expuestas a MMC, demostrando que el oncogén no interfiere la función del *core*, lo que indicaba que el defecto se localizaría en pasos posteriores a la monoubiquitinación de FANCD2 en la ruta de la AF/BRCA (Artículo 1; figura 3).

2. Estudio de aberraciones centrosómicas y cromosómicas en progenitores hematopoyéticos BCR-ABL1 en respuesta a agentes entrecruzantes del ADN.

La amplificación centrosómica es frecuente en todos los tipos de cáncer y correlaciona con la progresión maligna de la enfermedad (Fukasawa, 2005; Pihan et al., 2001; Skyldberg et al., 2001). Como ocurre en las células deficientes en BRCA1 (Xu et al., 1999), la presencia de aberraciones centrosómicas y la aneuploidía son dos características comunes de las células de LMC. De hecho, datos previos muestran que las aberraciones centrosómicas correlacionan directamente con la fase de la enfermedad y preceden a las alteraciones cromosómicas en células primarias de pacientes con LMC (Giehl et al., 2005).

Nalepa y col. han descrito recientemente que la señalización de la ruta de AF es importante para la segregación fidedigna de los cromosomas y la prevención de aneuploidía. De hecho, descubrieron que al menos 8 proteínas de AF se localizan en los centrosomas durante la mitosis (FANCA, FANCB, FANCE, FANCG, FANCL, FANCD1 y FANCN) y que éstas, son esenciales para el mantenimiento de un número de centrosomas normales en la célula y el punto de control del ensamblaje del huso en mitosis (Nalepa et al., 2013).

Más allá de sus funciones durante la citocinesis y la progresión del ciclo, los centrosomas también participan en la respuesta al daño o la replicación incompleta en el ADN (Hut et al., 2003; Sibon et al., 2000). Es más, se ha descrito conexión física y funcional entre el centrosoma y otros componentes de la maquinaria de reparación del

ADN, como NBS1 (Shimada et al., 2009), ATR (Griffith et al., 2008), ATM (Bourke et al., 2007; Zhang et al., 2007b), BRCA1 (Hsu and White, 1998; Ko et al., 2006), BRCA2/FANCD1 (Nakanishi et al., 2007) y PARP1 (Kanai et al., 2003). En este sentido, se ha descrito que la duplicación aberrante de los centrosomas puede ser inducida por distintos agentes genotóxicos, incluida la radiación ionizante (Sato et al., 2000). Por el contrario, Hut y col. demostraron que los centrosomas en lugar de amplificarse se fragmentan en respuesta al daño por MMC durante la mitosis, generando fracciones que contienen un único centriolo en la línea CHO deficiente en el control de ciclo en G2/M. Como resultado se generaban husos mitóticos multipolares, alteraciones en la segregación de los cromosomas y divisiones aberrantes (Hut et al., 2003). La aparición de centrosomas aberrantes en las células $CD34^+$ transducidas con BCR-ABL1 expuestas a MMC (Artículo 1; figura 6) se muestra en consonancia con resultados obtenidos en líneas de cáncer de mama que son sometidas a tratamiento con distintos agentes genotóxicos (D'Assoro et al., 2004). La presencia de esas aberraciones centrosómicas nada más expresarse el oncogén refuerza la hipótesis de que este fenotipo constituye un evento temprano en la transformación de las células de LMC.

La generación de centrosomas aberrantes en las células BCR-ABL1 es consistente con la inestabilidad cromosómica en estas células, una observación que es evidente tras la exposición a diferentes agentes que dañan el ADN, incluido las ROS, radiación ionizante y etopósido (Dierov et al., 2009; Koptyra et al., 2008). En este sentido, nuestro estudio muestra por primera vez la inducción de inestabilidad cromosómica en células $CD34^+$ transducidas con *BCR-ABL1* en respuesta al agente entrecruzante del ADN DEB (Artículo 1; figura 8), que se usa de forma clásica para el diagnóstico de la AF(Auerbach and Wolman, 1976). Aparte de la generación aberrante de centrosomas y alteraciones de reparación de la ruta de la AF durante la fase S, se ha descrito recientemente un nuevo mecanismo por el que la deficiencia de algunas proteínas de la ruta AF/BRCA (como FANCD2, FANCM o FANCI) induce inestabilidad cromosómica en la célula. Según estos estudios, FANCD2-Ub tendría un papel fundamental durante la mitosis, estabilizando los sitios frágiles y protegiendo los puentes ultrafinos que unen las cromátidas hermanas en respuesta al estrés replicativo o agentes como la Afidicolina, la MMC o la Hidroxiurea (Chan et al., 2009; Howlett et al., 2005 ; Naim and Rosselli, 2009; Vinciguerra et al., 2010). Sin embargo, no se ha estudiado hasta la fecha, si en células BCR-ABL1 existe una deficiente traslocación de FANCD2-Ub al cromosoma en mitosis, lo que impediría su localización en los extremos de los puentes

ultrafinos durante la anafase, para facilitar la resolución de los productos intermedios de replicación en las células BCR-ABL1.

3. Papel del supresor de tumores BRCA1 en las alteraciones centrosómicas y cromosómicas de las células BCR-ABL1.

El supresor de tumores BRCA1, cuyas mutaciones están asociadas a la aparición de cáncer de mama, tiene un papel esencial en la reparación del ADN, así como en la regulación de los sensores del ciclo celular, el control transcripcional y la remodelación de la cromatina (Narod and Foulkes, 2004). Diferentes observaciones sostienen la hipótesis de que uno de los mejores candidatos para interferir la traslocación de FANCD2 a la cromatina en células BCR-ABL1 es BRCA1. Primero, porque BRCA1 está regulado negativamente a nivel post-transcripcional por BCR-ABL1 (Deutsch et al., 2003); segundo, aunque BRCA1 no es esencial para la monoubiquitinación de FANCD2 (Vandenberg et al., 2003), es necesario para la unión de FANCD2 con la histona γH2AX en las horquillas de replicación bloqueadas (Bogliolo et al., 2007) y para la posterior formación de los *foci* después del daño en el ADN (Garcia-Higuera et al., 2001; Vandenberg et al., 2003) y tercero, las células *BRCA1*$^{-/-}$ comparten con las células de AF un fenotipo de inestabilidad cromosómica (Xu et al., 1999). Además, en los ensayos de ciclo celular, las células BCR-ABL1 tratadas con MMC no se acumulan en G2/M (Artículo 1; figura S1) a diferencia de lo que ocurre con las células de AF (Seyschab et al., 1995). Como las células deficientes en BRCA1 tienen un defecto en el punto de control del ciclo celular de G2/M (Xu et al., 1999) este fenotipo remarcaba de nuevo el papel de BRCA1 en la interferencia de la ruta de la AF en células BCR-ABL1.

Para clarificar los mecanismos potencialmente responsables de la represión de BRCA1 y por tanto, del defecto en la formación de *foci* de FANCD2 en células de LMC, se investigó el papel del proteosoma y la ruta de PI3K/AKT en la regulación post-traduccional de BRCA1. La ruta de señalización de PI3K/AKT está frecuentemente activada en cánceres humanos y también en células de LMC (Skorski et al., 1997). En este sentido, los resultados obtenidos en células primarias y líneas de cáncer de mama y ovario han mostrado que la onco-cinasa AKT1 es capaz de inhibir la formación de *foci* de BRCA1(Plo et al., 2008; Tonic et al., 2010). Concretamente en el presente estudio, los resultados mostraron que la inhibición de la ruta PI3K/AKT con LY294002 restauraba la formación de los *foci* de BRCA1 así como los de FANCD2 en las células CD34^{+} transducidas con *BCR-ABL1*. El mismo efecto se observó utilizando

el inhibidor de proteosoma MG132, indicando que esta molécula no sólo restaura la expresión de BRCA1 en células *BCR-ABL1*, como ya se había descrito previamente con Lactacistina (Deutsch et al., 2003), sino que restaura la aparición de *foci* de BRCA1 y FANCD2 en estas células (Artículo 1; figura 4). Los resultados en células BCR-ABL1 co-transducidas con un vector retroviral que portaba *BRCA1*, confirmaron que la expresión ectópica de BRCA1 revierte parcialmente la inhibición en la formación de *foci* de FANCD2 en células BCR-ABL1 (Artículo 1; figura 5).

Diversos estudios han demostrado que BRCA1 tiene un papel fundamental en la duplicación de los centrosomas. De hecho, BRCA1 se localiza físicamente en los centrosomas (Hsu and White, 1998) y su deficiencia en ratón produce amplificación centrosómica (Xu et al., 1999). El heterodímero BRCA1-BARD1 se activa por NBS1 y es capaz de actuar como un regulador negativo monoubiquitinando γ-TUBULINA, un componente esencial de la matriz pericentriolar, a través de su actividad ubiquitin-ligasa E3 (Shimada et al., 2009). La inhibición de la monoubiquitinación causa sobreduplicación de los centrosomas y una nucleación microtubular aberrante en líneas celulares derivadas de tejido mamario (Sankaran et al., 2005 ; Starita et al., 2004). Nuestros resultados demuestran por primera vez que la expresión ectópica de BRCA1 en células $CD34^+$ BCR-ABL1 disminuye el porcentaje de células que presentan amplificación centrosómica (Artículo 1; figura 6). Por tanto, podemos concluir que la disminución de la expresión de BRCA1 es al menos en parte responsable de la aparición de este fenotipo en células BCR-ABL1, y como ocurre durante el desarrollo de cáncer de mama (Lingle et al., 2002), la amplificación centrosómica puede dirigir la aparición de inestabilidad cromosómica en LMC.

Finalmente, resulta de particular importancia la observación de que la expresión ectópica de BRCA1 en células BCR-ABL1 disminuye el número de células con cromosomas aberrantes y, en mayor medida, de cromosomas multiaberrantes en respuesta a DEB (Artículo 1; figura 8). Esto sugiere que la expresión ectópica de BRCA1 podría ser capaz de restaurar la función reparadora de la ruta de la AF sobre las ICLs generadas en el ADN. Estos resultados aportan nueva información a los datos obtenidos por Deutsch y col. (Deutsch et al., 2003) que mostraban una disminución de la expresión de BRCA1 en células hematopoyéticas BCR-ABL1, y suponen la primera descripción de una alteración en la ruta de AF en células de LMC. En particular, nuestros resultados sugieren que la disminución en la expresión de BRCA1 impide la traslocación de FANCD2-Ub a los sitios de daño en el ADN y compromete la estabilidad genética de las células BCR-ABL1.

4. Análisis de la sensibilidad de progenitores hematopoyéticos BCR-ABL1 a agentes entrecruzantes del ADN. Posible papel dual de FANCD2-Ub en la supervivencia y la estabilidad genética de progenitores LMC.

Una de las características más relevantes de las células de AF y de células deficientes en BRCA1 es su marcada sensibilidad a agentes entrecruzantes del ADN (Auerbach and Wolman, 1976; Moynahan et al., 2001). Sin embargo, la resistencia a estos agentes en líneas celulares *BCR-ABL1* está bien documentada (Nieborowska-Skorska et al., 2006; Rink et al., 2007; Slupianek et al., 2005; Slupianek et al., 2002; Slupianek et al., 2001). En este trabajo se muestra que BCR-ABL1 confiere resistencia y también inestabilidad cromosómica en progenitores hematopoyéticos expuestos a agentes entrecruzantes del ADN (Artículo 1; figura 7 y 8). La observación de que BCR-ABL1 induce resistencia a los efectos letales de la MMC no es sorprendente, puesto que se conoce que uno de los mecanismos por los que BCR-ABL1 potencia la resistencia celular al estrés genotóxico se debe a la inhibición de las rutas pro-apóptoticas (Ghaffari et al., 2003; Horita et al., 2000; Keeshan et al., 2002; Mukhopadhyay et al., 2002; Neshat et al., 2000). A este respecto, diversos estudios han demostrado que BCR-ABL1 protege de la apoptosis mediada por el daño en el ADN de forma dosis-dependiente (Cambier et al., 1998; Keeshan et al., 2001) debido a la capacidad de la oncoproteína para regular la expresión y/o actividad de factores pro y anti-apoptóticos, a través de la señalización de las rutas de STAT5 (Horita et al., 2000), PI3K/AKT (Ghaffari et al., 2003) y RAS (Aichberger et al., 2005; Cortez et al., 1996). Alternativamente, Slupianek y col. proponen como mecanismo fundamental de resistencia a MMC y cisplatino, la fosforilación constitutiva en Y315 en BCR-ABL1 y el aumento de expresión de la recombinasa RAD51, mediada por BCR-ABL1 (Slupianek et al., 2001).

En Abril del 2011, cuatro meses después de la publicación del primer trabajo que compone esta tesis doctoral, Koptyra y col. mostraron un aumento de expresión de FANCD2-Ub en células $CD34^+$ de LMC y en líneas celulares BCR-ABL1 en respuesta a ROS y a la exposición a MMC (Koptyra et al., 2011). Los autores demostraron que la inhibición de la expresión o la monoubiquitinación de FANCD2 reducían la capacidad clonogénica de las células $CD34^+$ de LMC y retrasaba la leucemogénesis inducida por una línea linfobástica que expresa BCR-ABL1 después de un trasplante en ratones. Además, Koptyra y col. (Koptyra et al., 2011) mostraban que *BCR-ABL1* protege a las células del efecto letal de las DSB inducidas por ROS, lo que indicaba que FANCD2-Ub tiene un papel importante en la leucemogénesis inducida por BCR-ABL1. En sus

resultados no se describe ninguna alteración en la formación de *foci de* FANCD2, sino al contrario, muestran un aumento de éstos en la línea megacarioblástica Mo7e, proponiendo la estimulación de la ruta de la AF como consecuencia de la expresión del oncogén. Al mismo tiempo, argumentaban que la alteración que se describe en los *foci* de reparación en nuestro trabajo (Artículo 1) se debía a que la detección de los mismos, se había producido a tiempos demasiado largos, en los que el daño inducido por MMC ya estaría reparado en las células BCR-ABL1.

El análisis de la cinética de reparación de las DSB inducidas por MMC, mediante co-localización con la histona γH2AX, nos permitió confirmar en células de pacientes LMC y en $CD34^+$ de SCU transducidas con el vector retroviral BCR-ABL1 que la inhibición de los *foci* de FANCD2 es evidente en células que tienen un elevado número de DSB (Artículo 2; figura 1), reforzando nuestros resultados que demostraban la existencia de este tipo de alteración en la ruta. Nuestros resultados no descartan, sin embargo, que BCR-ABL1 esté aumentando la expresión de la forma FANCD2-Ub y que ésta tenga un papel en la supervivencia de las células de LMC, independientemente de su función en la estabilidad cromosómica para la que se requiere la formación de *foci* nucleares de FANCD2 (Garcia-Higuera et al., 2001). En este sentido, se conoce que algunas de las proteínas de la AF que componen el *core,* como son FANCC y FANCG, desempeñan un papel fundamental a través de su interacción con componentes citoplasmáticos implicados en el metabolismo redox, en la protección celular contra el estrés oxidativo (Cumming et al., 2001; Futaki et al., 2002; Koh et al., 1999). En relación a FANCD2-Ub, un estudio previo demuestra su interacción física y funcional con FOXO3a, uno de los reguladores más importantes de la respuesta al estrés oxidativo, formando un complejo que confiere resistencia celular específica a este tipo de daño provocado con H_2O_2, que no se induce con MMC ni con radiación ionizante (Li et al., 2010a). Asimismo Willers y col. publicaron en 2008, que la formación de *foci* de FANCD2 no constituye toda la función de la proteína, puesto que su formación no se requiere para inducir resistencia celular a daño oxidativo en el ADN, por lo que FANCD2 podría mediar esa resistencia con independencia de su función en la respuesta a daño en el ADN asociada a la replicación (Willers et al., 2008). Recientemente, Du y colaboradores, han aportado la última prueba que demuestra que la ruta de AF/BRCA no es lineal en respuesta al estrés oxidativo y a la reparación de la ICL. Para los autores, FANCD2-Ub se localizaría junto al factor remodelizador de la cromatina BRG1, protegiendo a los promotores de los genes antioxidantes del daño oxidativo inducido por H_2O_2, pero sin participar en su reparación (Du et al., 2012) . En definitiva, teniendo en cuenta los resultados en conjunto, proponemos un modelo

(Artículo 2; figura 2) en el que la función de FANCD2-Ub se encontraría disociada en células BCR-ABL1. Por una parte, el aumento en la expresión de FANCD2 y su monoubiquitinización en K561, facilitaría la supervivencia de las células de LMC al daño oxidativo y, por otra, la inhibición de la formación de *foci* de FANCD2, debido a la disminución en la expresión de BRCA1, contribuiría a la inestabilidad genética en estas células. Quedaría por dilucidar a través de qué ruta o rutas, FANCD2-Ub está induciendo supervivencia de las células de LMC sin participar en los focos de reparación. En este sentido, describimos por primera vez la formación del complejo FANCD2-FOXO3a (Artículo 3 figura 2e) en condiciones basales en células que expresan BCR-ABL1, en donde FOXO3a inmunoprecipita preferentemente con la fracción monoubiquitinada de FANCD2 (Artículo 3 figura 2e). Es importante resaltar que la interacción FANCD2-FOXO3a correlaciona directamente con la expresión de la proteínas antioxidantes SOD2, CATALASA o GLUTATIÓN-PEROXIDASA (Li et al., 2010a) y que la proteína antioxidante SESTRINA-3, cuyo gen *SESN-3*, es una diana transcripcional de FOXO3a y por tanto podría estar modulando los niveles de ROS en LMC (Hagenbuchner et al., 2012 ; Nogueira et al., 2008). En conjunto, postulamos que aunque FANCD2-Ub no está contribuyendo a la reparación en células que expresan BCR-ABL1, sí que tiene un papel en la detoxificación de la producción espontánea de ROS en la célula, lo que facilitaría el control del daño oxidativo y por tanto su supervivencia.

5. Análisis del patrón de expresión de los miRNAs 183-96-182 en las células que expresan BCR-ABL1 y su papel en el control del supresor de tumores BRCA1.

Numerosos trabajos han mostrado que la expresión de BCR-ABL1 puede alterar la respuesta al daño en el ADN, afectando a sensores/mediadores (Deutsch et al., 2003; Rink et al., 2007) y transductores (Dierov et al., 2004) del daño o a distintos efectores de la reparación (Deutsch et al., 2001; Salles et al., 2011; Slupianek et al., 2001). En lo que respecta a BRCA1, BCR-ABL1 puede disminuir su expresión, causando la desaparición del punto de control en mitosis (Wolanin et al., 2010) y como se ha descrito, un defecto evidente en la traslocación de FANCD2 a los *foci* de reparación (Artículo 1; figura 1 y 2), que compromete la función reparadora de la ruta de la AF. Sin embargo, se desconoce a través de qué mecanismos, el oncogén de fusión puede disminuir post-transcripcionalmente el supresor BRCA1 en células de LMC. La serina/treonina ATM es la cinasa prototípica que fosforila BRCA1 en respuesta al daño celular en el ADN causado por DSB. Sin embargo, no se han detectado mutaciones ni

cambios en su actividad debido a su interacción con BCR-ABL1 (Melo et al., 2001). Distintos trabajos en tumores sólidos esporádicos y líneas de cáncer de mama indican que uno de los candidatos para modular a la baja los niveles de BRCA1 en condiciones patológicas es la serina-treonina cinasa AKT, activada en aproximadamente el 30% de estos cánceres (Gonzalez et al., 2011; Guirouilh-Barbat et al., 2010; Plo et al., 2008; Plo and Lopez, 2009; Tonic et al., 2010; Xiang et al., 2011). En el presente trabajo, la reversión de la formación de *foci* de BRCA1 obtenida en células que expresan BCR-ABL1 mediante inhibidores de la ruta de la PI3K/AKT, es consistente con que la onco-cinasa AKT, sea parcialmente responsable de la disminución de la expresión de BRCA1 en LMC (Artículo 1; figura 4). Sin embargo, no existen evidencias de que la fosforilación de BRCA1 por AKT sea la responsable de su represión. Recientemente se han publicado otros mecanismos diferentes que también pueden estar mediando este efecto en distintas líneas celulares que no presentan el oncogén *BCR-ABL1* (Hammond-Martel et al., 2010; Laulier et al., 2011).

Es tentador, por tanto, especular con la posibilidad de que exista una ruta alternativa en células BCR-ABL1 para modular los niveles de BRCA1 mediante alguno de los reguladores post-transcripcionales mejor conocidos: los miARNs. En este sentido, el trabajo siguiente (Artículo 3) se centró en el estudio de aquellos miARNs que pueden unirse a la región 3' UTR de BRCA1, mediante análisis predictivo *in silico*, comparando los miARNs resultantes de este análisis con aquellos miARNs que tienen una expresión elevada en células de LMC. Como resultado de los análisis *in silico* se identificaron dos miembros del grupo miR-183-miR-96-miR-182, concretamente miR-183 y miR-96, que podían tener como diana BRCA1 (Artículo 3; figura 1a). Además, experimentos llevados a cabo en células de pacientes con LMC ya habían confirmado el aumento en la expresión del miR-96 (Agirre et al., 2008). De acuerdo con estas observaciones, el análisis del grupo miR-183-miR-96-miR-182 en progenitores hematopoyéticos de pacientes de LMC al diagnóstico mostró un aumento en la expresión de todos los componentes del grupo (Artículo 3; figura 1b), sugiriendo un papel relevante de estos miARNs en la enfermedad. De igual forma, los resultados también confirmaron un aumento de expresión en los tres componentes del grupo en células $CD34^+$ de SCU transducidas con BCR-ABL1 (Artículo 3; figura 1c).

En este caso, distintos miARNs podrían estar regulando la misma región 3' UTR de BRCA1 para asegurar la correcta regulación de un gen clave como éste en células de LMC. En relación a la respuesta al daño en el ADN, varios trabajos recientes han relacionado la expresión de componentes del grupo miR-183-miR-96-miR-182 con

dianas que participan en la reparación del ADN, incluido *BRCA1* en el caso de cáncer de mama y ovario (Liu et al., 2012; Moskwa et al., 2011 ; Xu et al., 2014b), *RAD51* y la TLS polimerasa *REV1* (Wang et al., 2012a). Uno de ellos (Moskwa et al., 2011), ha analizado en concreto la relación entre el tercer componente del grupo, miR-182, y BRCA1. Este trabajo confirma que la disminución en la expresión de BRCA1 en algunos tumores de mama es consecuencia del aumento en la expresión de miR-182. Las diferencias en la identificación *in silico* del tercer miembro de este grupo de miARN como regulador directo de BRCA1 se explican porque los autores usaron un algoritmo distinto, RNA22, que permite detectar interacciones miARN/diana poco conservadas entre especies (Moskwa et al., 2011). No obstante, los experimentos con luciferasa en el presente trabajo demostraron que el grupo completo de miARNs está regulando directamente la región 3' UTR de BRCA1 (Artículo 3; figura suplementaria 6a).

Si consideramos la relevancia de la expresión de BRCA1 en el mantenimiento de la estabilidad genética de la célula BCR-ABL1, como se ha descrito anteriormente en este trabajo (Artículo 1), el incremento en la expresión del grupo miR-183-miR-96-miR-182 en células que expresan BCR-ABL1 podría explicar la disminución en la expresión de BRCA1, y en consecuencia contribuir a su inestabilidad cromosómica y, por tanto, a la progresión hacia la crisis blástica de la LMC. En este sentido, los resultados obtenidos con vectores lentivirales que expresan ARN de interferencia contra los distintos miembros del grupo miR-183-miR-96-miR-182 no sólo han mostrado un incremento en la formación de *foci* de BRCA1 en células BCR-ABL1 (Artículo 3; figura 2b y 2c) sino que también aumentaron la proporción de células con *foci* de FANCD2 (Artículo 3, figura suplementaria 4), corroborando nuestros datos previos que indican la relevancia de los niveles de BRCA1 en la funcionalidad de la ruta de AF en células de LMC (Artículo 1). Estos resultados eran similares a los observados cuando se transdujeron células $CD34^+$ BCR-ABL1 con un vector retroviral que permite expresar una forma resistente a la regulación por miARNs de BRCA1, porque carece de región 3' UTR (Artículo 1; figura 5). Por otra parte, los efectos de la re-expresión de BRCA1 mediante la inhibición de los distintos componentes del grupo miR-183-miR-96-miR-182 se confirmaron también cuando se analizó la fragilidad cromosómica espontánea de células $CD34^+$ transducidas con BCR-ABL1. La reversión de la expresión de BRCA1, debido a la interferencia en la expresión del grupo miR-183-miR-96-miR-182, disminuyó la inestabilidad cromosómica en células $CD34^+$ BCR-ABL1 (Artículo 3; figura 3c). Y a la inversa, la expresión ectópica de este grupo de miARNs en estas células incrementó de manera marcada su inestabilidad cromosómica (Artículo 3; figura 3c). De forma sorprendente, la disminución de la fragilidad cromosómica y la

aparición de *foci* de BRCA1 no correlacionó con una disminución de los niveles de γH2AX en Fase S del ciclo celular, medido como marcador de DSB en el ADN cuando se interfirió el grupo miR-183-miR-96-miR-182 (Artículo 3 figura 3b). Estos resultados nos hicieron sospechar que la fragilidad cromosómica podría estar causada por la deficiente fidelidad del mecanismo de reparación implicado en la reparación de las DSBs, más que por cambios drásticos en el balance global de las mismas. Esta hipótesis se soporta en dos evidencias previas: Aunque se ha publicado que BCR-ABL1 estimula todos los mecanismos de reparación de DSB (HR, NHEJ y SSA) (Muvarak et al., 2012) generando una reparación no fidedigna e inestabilidad cromosómica, un estudio reciente (Chakraborty et al., 2012) ha postulado que la inestabilidad cromosómica en células de LMC en fase crónica se produce por un aumento del sistema de reparación de NHEJ clásico, que genera fusiones en el ADN que resultan en puentes anafásicos durante la mitosis. Por tanto, propone que la inestabilidad cromosómica en la LMC se produce a consecuencia de ciclos continuos del mecanismo rotura-fusión-puente (RFP). La segunda evidencia se basa en la participación de los componentes de la ruta AF/BRCA en cada uno de estos procesos, es decir, desplazando la reparación de DSB hacia la HR (Nakanishi et al., 2005) en fase S del ciclo y suprimiendo activamente los factores del NHEJ (Adamo et al., 2010; Pace et al., 2010), junto con sus funciones conocidas en mitosis, impidiendo la formación de puentes anafásicos (Naim and Rosselli, 2009) y el fallo citocinético (Vinciguerra et al., 2010). Es más, tanto BRCA1 como FANCD2 participan activamente en el procesamiento del extremo de la DSB, favoreciendo el inicio de la HDR en la fase S del ciclo (Escribano-Diaz et al., 2013; Murina et al., 2014; Unno et al., 2014). En estas condiciones se ha observado que BRCA1 es capaz de desplazar físicamente a la proteína inductora del NHEJ 53BP1, del *foci* de reparación (Chapman et al., 2012). De hecho, se ha demostrado que niveles bajos de BRCA1 pueden provocar la inducción de un NHEJ tóxico durante el proceso de reparación de la DSB asociado a la replicación, porque no puede competir con el complejo 53BP1-RIF1 durante el procesamiento del extremo de la doble rotura (Bunting et al., 2010; Cao et al., 2009). Este mecanismo puede favorecer la aparición de ciclos RFP. Por tanto, es tentador especular con la posibilidad de una posible contribución de la ruta AF/BRCA sobre los ciclos RFP también en las DSBs espontáneas generadas por ROS.

Los primeros resultados a este respecto fueron consistentes con esta hipótesis, puesto que la interferencia de todos los miembros del grupo de miR-183-miR-96-miR-182 causó la disminución en la proporción de células en fase S con *foci* de 53BP1 (Artículo 3 figura 3e). En esta ocasión, estos datos sí correlacionaron con los niveles de

expresión de BRCA1 y la fragilidad cromosómica que presentan estas células, lo que sugiere que la expresión del grupo miR-183-miR-96-miR-182 podría estar participando en el control de la actividad de NHEJ durante la fase S. Curiosamente, la expresión ectópica del grupo miR-183-miR-96-miR-182 aunque inducía en células una mayor fragilidad cromosómica (Artículo 3 figura 3c) y niveles prácticamente inexistentes de BRCA1 y FOXO3a (Artículo 3 figura 2a), éstos correlacionaban con una disminución marcada de los niveles de γH2AX (Artículo 3 figura 3b). Este resultado nos indujo a pensar que se podría estar produciendo en estas células un defecto en la señalización del daño en el ADN. La serina/treonina cinasa que fosforila H2AX cuando se ha producido la DSB es ATM (Burma et al., 2001). Nuestros resultados indican que la actividad de ATM se ve comprometida cuando se fuerza la expresión en las células del grupo miR-183-miR-96-miR-182 (Artículo 3 figura 3d). A diferencia de γH2AX, 53BP1 no requiere de la fosforilación de ATM para localizarse en el foco de daño (Schultz et al., 2000; Ward et al., 2003), pero ésta si es esencial para la reparación por NHEJ, porque ATM activado es necesario para el funcionamiento de 53BP1 (Rappold et al., 2001) y también para la activación de la DNA-PKcs (Chen et al., 2007). Por tanto, hipotetizamos que en estas células, 53BP1 puede resultar un marcador válido de las DSBs producidas en el ADN pero probablemente ni el NHEJ ni la HR puedan activase para reparar ese daño, lo que redundaría en un incremento en la cantidad de roturas cromosómicas. En consonancia con la participación de la ruta AF/BRCA en la estabilidad cromosómica durante la mitosis, también cabe destacar que en células BCR-ABL1, nuestros resultados demuestran que la desaparición de BRCA1 mediada por la expresión ectópica del grupo de miR-183-miR-96-miR-182, aumentó significativamente el porcentaje de células bi- o multinucleadas (Artículo 3 figura suplementaria 5), provocando la aparición de puentes nucleoplásmicos y células con micronúcleo, reflejo de la existencia de mitosis aberrantes que pueden comprometer la supervivencia o inducir el proceso de senescencia celular.

En conjunto, estos resultados demuestran por primera vez la implicación de los diferentes componentes del grupo miR-183-miR-96-miR-182, como parte de un nuevo mecanismo que disminuye la expresión de BRCA1 y genera inestabilidad cromosómica en células $CD34^+$ que expresan BCR-ABL1.

6. Consecuencias de la expresión del grupo de miARNs 183-96-182 en la proliferación y la capacidad clonogénica de progenitores hematopoyéticos BCR-ABL1.

Una amplia variedad de estudios ha demostrado el aumento de la expresión del grupo de miARNs 183-96-182 en distintas neoplasias (Guttilla and White, 2009; Hannafon et al., 2011; Lehmann et al., 2010; Li et al., 2010b; Lowery et al., 2010; Navon et al., 2009; Sarver et al., 2009; Segura et al., 2009; Wang et al., 2008; Yamada et al., 2010) y de su función como onco-miARNs (Li et al., 2010b; Lin et al., 2010; Sarver et al., 2010). La regulación de la expresión de este grupo de miARNs no se conoce del todo, aunque se ha caracterizado la existencia de un único pri-miARN policistrónico controlado por un promotor que regula la expresión de sus tres componentes (Weston et al., 2006; Xu et al., 2007). Strittrich y col. (Stittrich et al., 2010) encontraron sitios de unión conservados para el factor de transcripción STAT5 en regiones intergénicas del grupo y demostraron en linfocitos T de ratón, que la expresión de al menos miR-182 estaba regulada directamente por este factor de transcripción. Estudios recientes también han relacionado la inducción de la expresión del miR-182 con los factores de transcripción SMAD, a través de la ruta de TGFβ (Song et al., 2012) y β-CATENINA (Chiang et al., 2013). En este sentido, nuestros resultados mostraron que la inhibición de STAT5 reducía la expresión de todos los componentes del grupo de miR-183-miR-96-miR-182 en células humanas que expresan BCR-ABL1 (Artículo 3 figura suplementaria 1), corroborando los resultados obtenidos previamente (Stittrich et al., 2010). Aparte de BRCA1, se conoce que otros supresores de tumores clave de la ruta PI3K/AKT/mTOR, son diana de este grupo de miARNs (Guttilla and White, 2009; Segura et al., 2009). En conjunto, estas observaciones sugerían que BCR-ABL1, a través de la activación constitutiva de STAT5 (Shuai et al., 1996), y por tanto del aumento en la expresión del grupo miR-183-miR-96-miR-182, podría controlar algunos de los supresores de tumores de la ruta PI3K/AKT/mTOR, generando un efecto redundante a la activación de esta ruta que no ha sido descrito previamente. Por esta razón, en los siguientes experimentos realizados en nuestro trabajo, estudiamos el efecto de la alteración en la expresión del grupo de miARNs sobre la proliferación y la capacidad clonogénica de células $CD34^+$ de SCU transducidas con BCR-ABL1.

Los primeros resultados indicaron que la interferencia del miR-183 y en menor medida, la de miR-182, disminuyen la proliferación y la capacidad clonogénica de progenitores hematopoyéticos $CD34^+$ transducidos con BCR-ABL1 (Artículo 3; figura 4a y b), sugiriendo por primera vez el papel del miR-183 como un onco-miARN en estas

células. En cuanto a las dianas de miR-183, se conoce que este miARN induce la modulación a la baja de *PDCD4* en células de carcinoma hepatocelular (Li et al., 2010b). PDCD4 actúa como un inhibidor de la traducción (Cmarik et al., 1999; Yang et al., 2003a; Yang et al., 2003b), que en células de LMC está regulado por mTOR (Carayol et al., 2008). Por tanto, el aumento en la expresión del miR-183 podría estar contribuyendo a la disminución de la expresión de PDCD4 en células BCR-ABL1. Se conoce que miR-182 y miR-183 también son capaces de regular EGR1 a nivel post-transcripcional (Hannafon et al., 2011; Sarver et al., 2010). EGR1 puede actuar como supresor tumoral; su activación está regulada por la ruta PI3K/AKT (Cabodi et al., 2009), y se ha descrito que niveles disminuidos de EGR1 correlacionan directamente con transformación celular y la formación de tumores (Ferraro et al., 2005; Liu et al., 1996). Es más, EGR1 controla directamente la transcripción del supresor de tumores PTEN (Virolle et al., 2001), luego el miR-183 puede indirectamente regular los niveles de PTEN a través de EGR1 cooperando en la progresión tumoral.

En LMC, la expresión de PTEN está disminuida y se comporta como un supresor tumoral (Peng et al., 2010b). Aunque se ha postulado que la represión de PTEN por BCR-ABL1 se debe al control transcripcional por p53 y la lipooxigenasa ALOX5 (Peng et al., 2010a; Peng et al., 2010b), en el siguiente conjunto de resultados se investigó si el grupo de miR-183-miR-96-miR-182 podía modular los niveles de PTEN.

Los resultados *in silico* predecían que *PTEN* podía ser diana directa del grupo de miR-183-miR-96-miR-182. En consistencia con ello, la interferencia de estos miARNs produjo un aumento considerable de los niveles de PTEN en células BCR-ABL1 (Artículo 3; figura 6e). Los ensayos de luciferasa sin embargo, demostraron que esta interacción no es directa, (Artículo 3; figura suplementaria 9a) sugiriendo que en células BCR-ABL1 la interacción miR-183/PTEN también podría estar mediada por EGR1. No obstante, no es descartable que los niveles de expresión de PTEN estén regulados de algún modo por BRCA1, puesto que la re-expresión de BRCA1 que se produce mediante la inhibición de todos los componentes del grupo miR-183-miR-96-miR-182, correlaciona con un aumento en PTEN (Artículo 3 figura 6e) y la expresión ectópica de *BRCA1* restaura la expresión de PTEN en células BCR-ABL1 (Artículo 3; figura suplementaria 9b). Resultados posteriores mostraron que el control de la expresión de PTEN por BRCA1 no se produce directamente vía regulación epigenética de su promotor (Artículo 3 figura suplementaria 9c y 9d). Es más, los resultados sugieren que esta modulación podría hacerse de nuevo a través de EGR1, puesto que la expresión ectópica de BRCA1 también restaura la expresión de EGR1 en células

BCR-ABL1 (Artículo 3 figura suplementaria 9e). Este grupo de experimentos muestran por primera vez que la represión de PTEN en células BCR-ABL1 puede deberse tanto a la reducción de EGR1, como diana directa del grupo de miR-183-miR-96-miR-182 de expresión incrementada en células BCR-ABL1, como por la subsiguiente reducción de BRCA1.

En conjunto, estos resultados indican que el grupo de miR-183-miR-96-miR-182 puede actuar como un onco-miR en células BCR-ABL1, inhibiendo supresores de tumores importantes en la LMC y que su inhibición podría contribuir a atenuar la leucemogénesis en pacientes con esta patología.

7. Modulación del supresor de tumores FOXO3a por el grupo de miR-183-miR-96-miR-182 y su relación con la acumulación de ROS.

Otras de las dianas relevantes de este grupo de miARNs son las proteínas FOXO. La subfamilia FOXO desempeña un papel importante como efectores de la ruta PI3K/AKT en diversas funciones celulares que incluyen la parada del ciclo celular, la resistencia celular al estrés, la apoptosis, la diferenciación y el metabolismo (Greer and Brunet, 2005). AKT inactiva FOXO1, FOXO3, FOXO4 por fosforilación y facilita su traslocación desde el núcleo hasta el citoplasma, donde se ubiquitinan y se degradan por el proteosoma (Brunet et al., 1999; Brunet et al., 2002). A la inversa, en respuesta a un estrés, la cinasa JUN N-terminal (JNK) o la cinasa MST-1 fosforilan las proteínas FOXO, induciendo su transporte al núcleo y su consiguiente activación transcripcional (Essers et al., 2005; Lehtinen et al., 2006). Las proteínas FOXO activadas se unen a los promotores de los genes de la manganeso superóxido dismutasa (SOD2) y la CATALASA, dos enzimas clave para la detoxificación oxidativa en mamíferos (Balaban et al., 2005; Kops et al., 2002) o al del gen de la proteína antioxidante SESTRINA-3 (*SESN3*) (Nogueira et al., 2008). Es importante resaltar que ante un estímulo estresante como el estrés oxidativo, la célula puede desplazar los efectos reguladores negativos de los factores de crecimiento sobre la activación de los factores FOXO, sugiriendo que entre las funciones más importantes de estas proteínas se encontraría la protección contra el estrés del ambiente sobre la célula (Brunet et al., 2004; Wang et al., 2005). En este sentido, Liu y col. (Liu et al., 2005) demostraron que varios estímulos apoptóticos, como la deprivación de suero, el tratamiento con una toxina mitocondrial o el daño en el ADN causado por etopósido en líneas de cáncer de mama, pueden activar distintos mecanismos pro-supervivencia mediante la inducción de la expresión de FOXO3a mediada por ROS.

Aunque en células de LMC está bien establecido que FOXO3a se comporta como un supresor de tumores (Brunet et al., 1999; Brunet et al., 2002), y que BCR-ABL1 a través de la activación de la ruta de la PI3K/AKT puede inhibir la función pro-apoptótica de FOXO3a (Ghaffari et al., 2003), resultados recientes indican que la expresión de FOXO3a es esencial para el mantenimiento de las células madre leucémicas durante la FC-LMC (Hurtz et al., 2011; Naka et al., 2010). Como FOXO3a es un factor determinante en la respuesta al estrés oxidativo (Huang and Tindall, 2007; Tsai et al., 2008) y dado que su interacción con FANCD2-Ub es necesaria para responder a este tipo de daño (Li et al., 2010a), especulamos con la posibilidad de que en los progenitores hematopoyéticos BCR-ABL1, donde las alteraciones en el flujo de la cadena mitocondrial introducen altos niveles de ROS (Nieborowska-Skorska et al., 2012), el mantenimiento de niveles nucleares limitados de FOXO3a inhibiría su función pro-apoptótica clásica mediada por BIM (Essafi et al., 2005) y TRAIL (Ghaffari et al., 2003), pero son suficientes para contribuir a la resistencia celular, comprometida por el incremento de daño oxidativo en el ADN, como consecuencia de la expresión de BCR-ABL1. De hecho, la interferencia del grupo de miR-183-miR-96-miR-182 en estas células fue capaz de disminuir la cantidad de ROS (Artículo 3 figura 3a). Estos datos correlacionaron con un incremento en el rol antioxidativo de la interacción FANCD2-FOXO3a (Artículo 3 figura 2f) y con la aparición de los *foci* de reparación conectando la ruta de AF/BRCA. Por el contrario, la eliminación de la interacción mediante la expresión ectópica de grupo del miR-183-miR-96-miR-182 no sólo aumentó los niveles de ROS sino que incrementó la inestabilidad cromosómica en las células BCR-ABL1. Se ha reconocido una función dual muy parecida a la deacetilasa dependiente de NAD SIRT1, promoviendo resistencia al estrés oxidativo e inhibiendo la muerte celular inducida por FOXO3a (Brunet et al., 2004). BCR-ABL1 activa la expresión de SIRT1 a través de la ruta de STAT5 en progenitores hematopoyéticos (Yuan et al., 2012), pero se ha demostrado que su expresión induce una reparación deficiente y la adquisición de nuevas mutaciones genéticas que contribuyen a la resistencia de la célula de LMC a inhibidores de tirosina cinasas (Wang et al., 2012b). Estos resultados sugieren de nuevo que las células BCR-ABL1 podrían activar mecanismos por los que se reclutarían efectores que participan en la supervivencia celular frente al estrés oxidativo, a expensas de la aparición de deficiencias en la reparación y el aumento de su inestabilidad genética. En consonancia con nuestros datos, un trabajo reciente ha correlacionado la activación de STAT5 con la producción de ROS en células de LMC (Warsch et al., 2012), por lo que proponemos la inducción en la expresión del grupo de miR-183-miR-96-miR-182 por STAT5, como posible mecanismo de acumulación de ROS en la enfermedad. Paralelamente, trabajos previos también han asignado a

BRCA1 un papel en la respuesta celular al estrés oxidativo (Bae et al., 2004; Saha et al., 2009), aumentando los niveles de expresión de genes antioxidantes mediante la regulación directa del factor de transcripción NRF2 (Bae et al., 2004 ; Gorrini et al., 2013).

Diferentes estudios han descrito la implicación de miR-96 y miR-182 en la regulación de la expresión de *FOXO1* y *FOXO3a* en cáncer de mama (Guttilla and White, 2009) y en el control de la metástasis en melanoma (Segura et al., 2009). Los resultados del presente trabajo también han confirmado la interacción entre el grupo miR-183-96-182 con la región 3'UTR de *FOXO3a* (Artículo 3; figura suplementaria 6b).

8. Análisis de la inducción de apoptosis y senescencia en los progenitores hematopoyéticos BCR-ABL1 tras la expresión ectópica del grupo miR-183-96-182. Descripción del mecanismo de senescencia inducida por BCR-ABL1. Consideraciones para la inducción de senescencia como terapia anti-tumoral en LMC.

La expresión ectópica del grupo de miARNs 183-96-182 en células $CD34^+$ de SCU transducidas con BCR-ABL1 redujo drásticamente la proliferación y clonogenicidad de estas células sin afectar a sus respectivos controles sanos (Artículo 3; figura 5 a-d). Los resultados de los ensayos de Anexina V (Articulo 3; figura 5e) y actividad β-galactosidasa (Artículo 3; figura 6a y 6b) en los cultivos de las células BCR-ABL1 revelan que, en las células transducidas con el grupo de miARNs 183-96-182 existe un incremento en la apoptosis, además de un marcado y significativo aumento de la senescencia celular comparado con su control. La senescencia en estas células no es sorprendente, puesto que trabajos previos han demostrado en distintos modelos la relación entre la falta de expresión de todas las dianas controladas por los miARNs 183-96-182 estudiadas en este trabajo, BRCA1 (Cao et al., 2003; Santarosa et al., 2009), FOXO3a (Kyoung Kim et al., 2005) y PTEN (Chen et al., 2005), con la aparición de senescencia prematura. Más aún, se ha publicado que al menos dos componentes del grupo, miR-182 y miR-183, incrementan su expresión durante el proceso de senescencia prematura inducida por estrés (SIPS) (Li et al., 2009).

La senescencia celular es un mecanismo fisiológico para impedir la proliferación de las células tumorales. Es una respuesta al estrés que se caracteriza por la inhibición de la proliferación, que puede volverse irreversible e independiente del estímulo estresante inicial. Entre estos estímulos se encuentran la señalización a través de proteínas oncogénicas, el daño oxidativo o el daño persistente en el ADN (Collado et al., 2007).

En relación a la senescencia inducida por oncogén (OIS) clásica, se ha observado que está asociada a una respuesta hiperproliferativa celular y a la activación de la respuesta al daño en el ADN (DDR) dependiente de ATM/ATR, que se refleja en la formación de *foci* de daño en el ADN asociado a la senescencia (SAHF) de γH2AX (Nardella et al., 2011). En LMC, Wajapeyee y col. demostraron que BCR-ABL1 no induce una senescencia asociada a DDR en progenitores hematopoyéticos de ratón. En el mismo trabajo, detectaron menor activación de la cinasa ATM y postularon que la señalización vía p38MAPK está implicada en el disparo de la senescencia en esas células (Wajapeyee et al., 2010). Nuestros resultados corroboraron en células progenitoras humanas la inexistencia de hiperproliferación previa a la aparición de senescencia y la ausencia de SAHF específicamente en células senescentes (Artículo 3 figuras 5c y 6c), con lo que no se ajustaba a las características de la OIS típica inducida por formas oncogénicas de BRAF o RAS previamente descritas (Dhomen et al., 2009; Serrano et al., 1997). Estos datos, junto con la ausencia de activación en ATM (Artículo 3 figura 3d) y la desaparición de PTEN cuando se expresa ectópicamente el grupo miARNs 183-96-182 (Artículo 3 figura 6e) sugirieron que podríamos estar ante un mecanismo de senescencia inducido por pérdida de PTEN (PICS). PICS es un mecanismo de senescencia sin hiperproliferación, que se induce en un período corto de tiempo, en ausencia de SAHF e independiente de activación por ATM/ATR, por lo que los progenitores BCR-ABL1 recapitularon fielmente sus características (Figura 18). Este mecanismo de senescencia fue descrito inicialmente en cáncer de próstata y células normales en el laboratorio de P.P. Pandolfi (Alimonti et al., 2010; Chen et al., 2005).

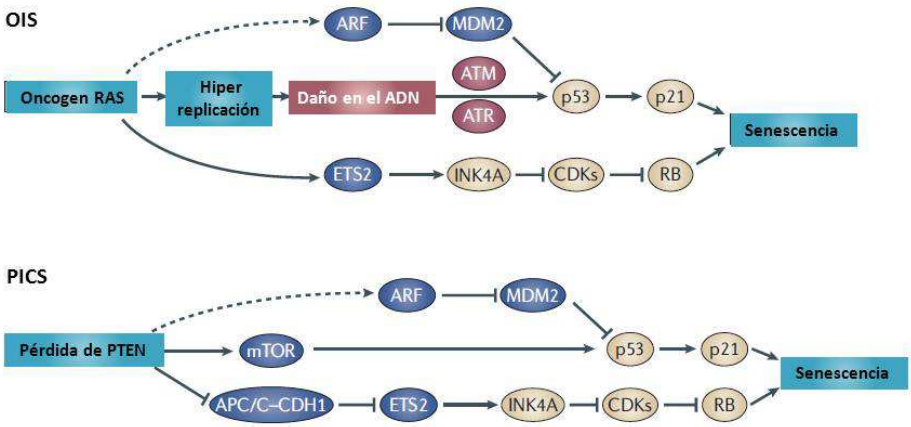

Figura 18: Senescencia inducida por oncogén (OIS) y senescencia inducida por pérdida de PTEN (PICS). Modificada de Nardela y col. (Nardella et al., 2011).

Estudios previos han demostrado que la deleción de *PTEN* produce una depleción en el compartimento de CMHs de ratón, aunque el papel de la senescencia en esta respuesta no se había demostrado (Lee et al., 2010; Yilmaz et al., 2006; Zhang et al., 2006). En este trabajo (Artículo 3) se asocia por primera vez la pérdida de PTEN con la aparición de senescencia en células progenitoras hematopoyéticas BCR-ABL1. Además, proponemos un nuevo mecanismo vía STAT5/miARNs 183-96-182/pérdida de PTEN para la inducción de esta senescencia. Paralelamente, Nogueira y col. (Nogueira et al., 2008) demostraron que la fosforilación de AKT, que se activa por la pérdida de PTEN, inhibe FOXO3a produciendo una acumulación de ROS, que finalmente es responsable del disparo de senescencia en las células. En nuestros experimentos, la descripción de que el grupo miARNs 183-96-182 controla *PTEN* y *FOXO3a* y que existe correlación con la acumulación de ROS sugiere la existencia de una nueva ruta alternativa para la inducción de senescencia. Esta nueva señal, que puede estar contribuyendo a la OIS en células BCR-ABL1 en estadios tempranos de la enfermedad, puede ser potenciada a través dela sobre-expresión del grupo de miARNs 183-96-182.

En este contexto oncogénico pre-tumoral, donde el umbral para el disparo de la senescencia es bajo, la eventual desaparición de FOXO3a y por tanto, el aumento de estrés oxidativo en estas células (Artículo 3 figura 3a), combinado con la eliminación de BRCA1 y PTEN podrían estar contribuyendo no sólo al aumento de la inestabilidad cromosómica en dichas células, sino también a los cambios drásticos en proliferación/clonogenicidad debidos a la inducción de PICS y apoptosis en las células $CD34^+$ BCR-ABL1, cuando se fuerza la expresión del grupo de miARNs. A la luz de estos resultados, y dado que el grupo de miARNs 183-96-182 controla varios de los principales supresores de tumores que están siendo regulados en las células BCR-ABL1, este grupo de miRNAs se propone como potencial diana terapéutica en LMC.

A pesar del posible potencial oncogénico del grupo de miARNs 183-96-182, su sobre-expresión puede tener una explotación terapéutica induciendo apoptosis o PICS. La terapia pro-senescente se ha revelado recientemente como estrategia para combatir la génesis de tumores sólidos *in vivo* (Nardella et al., 2011). En particular, desde una perspectiva terapéutica la PICS ofrece algunas ventajas sobre la OIS clásica. Primero, no requiere una fase hiperproliferativa ni DDR, luego puede ser inducida en tiempos más cortos disminuyendo la inestabilidad genética y el riesgo de adquisición de mutaciones secundarias. Segundo, la ausencia de replicación en el ADN sugiere que puede ser inducida en células quiescentes, como las células iniciadoras de la

leucemia, lo que resulta muy relevante en LMC. Tercero, PICS es una respuesta dependiente de p53 y su activación es a nivel traduccional, por lo que se podría inducir potencialmente inhibiendo reguladores negativos de p53, como HDM2. Atendiendo a nuestros resultados, es importante tener en cuenta que aunque la DDR no está activada en las células senescentes, sí existe daño en el ADN detectado por la aparición de 8-oxodG y la localización de 53BP1 en *foci* nucleares (Artículo 3 figuras 6d y suplementaria 8). A nuestro parecer, esta es la primera observación de daño por DSBs en la senescencia PICS, que se ha medido habitualmente con resultados negativos por el método de la cola de cometa o mediante *foci* de γH2AX (Wajapeyee et al., 2010). Por esta razón, puede producirse la aparición de nuevas mutaciones que produzcan un escape de la senescencia en la célula o, dado que la PICS es una respuesta dependiente de p53, la generación de presión selectiva sobre p53; mutado frecuentemente en estadios avanzados de la LMC (Feinstein et al., 1991), permitiendo la selección de clones malignos. Dado que PICS ocurre *in vivo* en estadios pre-neoplásicos (Chen et al., 2005), estamos llevando a cabo actualmente experimentos *in vivo* sobre-expresando el grupo de miARNs 183-96-182, para confirmar la seguridad de la inducción de PICS como una posibilidad terapéutica en estadio leucémico.

9. Consideraciones finales.

El análisis global de los resultados obtenidos en esta memoria pone de manifiesto el papel pivotante de la ruta de AF/BRCA en la generación de la inestabilidad genética de células LMC (Figura 19). Concretamente, en este trabajo se describe un nuevo mecanismo, alternativo al mediado por la activación de ruta PI3K/AKT, por el cual la expresión de BCR-ABL1, a través del aumento en la expresión del grupo de miARNs 183-96-182, disminuiría los niveles de tres supresores de tumores clave en la LMC: *BRCA1*, *FOXO3a* y *PTEN*. Nuestros resultados demuestran que la deficiencia en BRCA1 inhibe la ruta de la AF/BRCA, y por tanto su función de reparación celular de las ICL durante la fase S, así como la regulación correcta de sus centrosomas durante la mitosis en repuesta al daño en el ADN. La disminución de los niveles de FOXO3a y BRCA1 provoca un aumento del estrés oxidativo y la aparición de inestabilidad cromosómica que, junto al bloqueo apoptótico inducido por el oncogén, permite la supervivencia de progenitores BCR-ABL1 con alteraciones genéticas que serían incompatibles con la supervivencia de células que no expresen la oncoproteína, lo que puede promover la acumulación de mutaciones necesarias para la progresión a la CB-LMC.

La expresión forzada de este grupo de miARNs es capaz de alterar el fenotipo de los progenitores BCR-ABL1 induciendo senescencia prematura por pérdida de PTEN de manera específica en estas células, por lo que pueden suponer una diana potencial para futuras terapias contra la LMC.

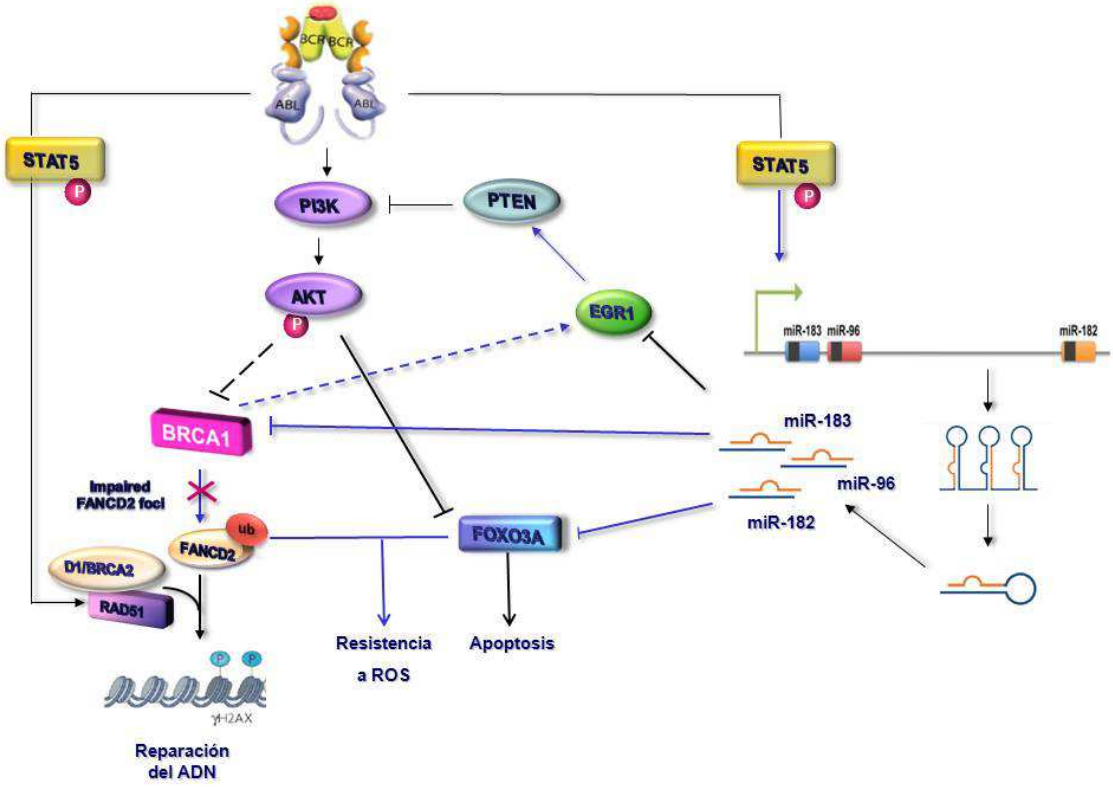

Figura 19: Diagrama esquemático ilustrativo de los principales hallazgos de esta memoria relacionados con la regulación del grupo de miARNs 183-96-182 y sus dianas moleculares así como sus implicaciones en el fenotipo de células que expresan BCR-ABL1. Las interacciones nuevas demostradas en este trabajo se reflejan en azul oscuro. Las líneas discontinuas representan interacciones indirectas.

IX. CONCLUSIONES

1. Los progenitores hematopoyéticos de pacientes con LMC presentan una alteración en la ruta de la AF/BRCA que consiste en una deficiente capacidad para formar *foci* nucleares de reparación del ADN que contienen la proteína FANCD2.

2. Los progenitores hematopoyéticos con la translocación *BCR-ABL1* muestran inestabilidad cromosómica y alteraciones en el número de centrosomas en respuesta a agentes entrecruzantes del ADN.

3. El supresor de tumores BRCA1 es esencial para la formación de *foci* de FANCD2, así como para el mantenimiento de la estabilidad centrosómica y cromosómica de las células BCR-ABL1.

4. A pesar de la disfunción en la ruta de AF/BRCA, BCR-ABL1 confiere a los progenitores hematopoyéticos resistencia a agentes entrecruzantes del ADN.

5. Los progenitores hematopoyéticos de pacientes con LMC muestran una expresión incrementada del grupo de miRNAs 183-96-182 que está mediada por BCR-ABL1.

6. En células BCR-ABL1, el incremento en la expresión de miRNAs 183-96-182 constituye un nuevo mecanismo que induce inestabilidad cromosómica y acumulación de especies reactivas de oxígeno (ROS) a través de la disminución de los niveles de *BRCA1* y *FOXO3a*.

7. Las alteraciones en la expresión del grupo de miARNs 183-96-182 ejercen un impacto marcado y específico en la proliferación y la capacidad clonogénica de progenitores hematopoyéticos que expresan BCR-ABL1, regulando los supresores de tumores *BRCA1*, *PTEN* y *FOXO3a*.

8. La expresión ectópica del grupo miR-183-96-182 induce senescencia prematura en progenitores hematopoyéticos BCR-ABL1.

9. El mecanismo de senescencia prematura mediado por el grupo miR-183-96-182 en progenitores BCR-ABL1 recapitula las características de la senescencia celular inducida por pérdida de PTEN.

Tomados en su conjunto, los resultados presentados en esta memoria ponen de manifiesto por primera vez la participación de la ruta de la AF/BRCA en la inestabilidad

genética de progenitores hematopoyéticos de pacientes con LMC y evidencia la existencia de nuevos mecanismos de regulación epigenéticos que pueden estar implicados en la evolución de la enfermedad, y por tanto resultar de utilidad para el desarrollo de nuevas terapias contra la LMC.

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XI. ANEXO 1

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In vitro sensitivity of granulo-monocytic progenitors as a new toxicological cell system and endpoint in the ACuteTox Project

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ABSTRACT

The ACuteTox Project (part of the EU 6th Framework Programme) was started up in January 2005. The aim of this project is to develop a simple and robust *in vitro* strategy for prediction of human acute systemic toxicity, which could replace animal tests used for regulatory purposes. Our group is responsible for the characterization of the effect of the reference chemicals on the hematopoietic tissue. CFU-GM assay based on the culture of human mononuclear cord blood cells has been used to characterize the effects of the selected compounds on the myeloid progenitors. Previous results have shown the relevance of the CFU-GM assay for the prediction of human acute neutropenia after treatment of antitumoral compounds, and this assay has been recently approved by the ECVAM's Scientific Advisory Committee. Among the compounds included in the study there were pharmaceuticals, environmental pollutants and industrial chemicals. Eleven out of 55 chemicals did not show any cytotoxic effect at the maximum concentration tested. The correlation coefficients of CFU-GM IC50, IC70 and IC90 values with human LC50 values (50% lethal concentration calculated from time-related sublethal and lethal human blood concentrations) were 0.4965, 0.5106 and 0.5142 respectively. Although this correlation is not improve respect to classical *in vitro* basal cytotoxicity tests such as 3T3 Neutral Red Uptake, chemicals which deviate substantially in the correlation with these assays (colchicine, digoxin, 5-Fluorouracil and thallium sulfate) fitted very well in the linear regression analysis of the CFU-GM progenitors. The results shown in the present study indicate that the sensitivity of CFU-GM progenitors correlates better than the sensitivity of HL-60 cells with human LC50 values and could help to refine the predictability for human acute systemic toxicity when a given chemical may affect to the hematopoietic myeloid system.

Introduction

The objectives of toxicology studies are the identification of potentially dangerous toxicants, so that human exposure can be prevented or controlled, and the provision of information relevant for undertaking risk-benefit analyses and for conducting clinical trials. *In vivo* toxicology studies are generally conducted in at least two different species, employing various dosing schedules. Any *in vitro* test which can refine the safety margins by reducing the toxicological uncertainties underlying laboratory animal/human extrapolations would be of great benefit, since it would provide a more scientific and rational basis for calculating clinical dosages and for setting human exposure limits. ACuteTox, which started up in January 2005, is an integrated project under the EU 6th Framework Programme, entitled "Optimisation and Pre-Validation of an *In Vitro* Test Strategy for Predicting Human Acute Toxicity". It is an ambitious project involving 35 partners organized in nine work packages (WP) (Clemedson et al., 2007). WP1 was responsible for the collection, from the literature, of *in vivo* human and animal data, over 2800 human cases and 2200 LD50 values respectively. WP2 defined the protocols and acceptance criteria for obtaining the *in vitro* data (Clothier, 2007). WP3 plays a central role in dealing with the iterative amendment of testing strategies, establishing a web-based database platform for central management of all data contributions, and performing the statistical analysis of the data, in order to identify outliers and those chemicals for which additional *in vitro* assays are required. All the Standard Operating Procedures (SOPs) produced and the results obtained are included in the database of the project, called Acutoxbase (Clemedson et al., 2007). It is so far accessible only to the participants of the project, but after its finalization, it will be opened to a larger circle of users (toxicologist, clinicians, researchers, regulators, etc.). Acutoxbase will serve as an excellent source of diligently compiled, good quality *in vivo* and *in vitro* data, as well as a wide collection of SOPs critically revised by experts in the respective fields (Kinsner-Ovaskainen et al., 2009). The influence of a range of parameters including biokinetics, biotransformation and target organ toxicity will be determined through *in vitro* testing and kinetic modeling. Thus, WP4 has implemented new cell systems and innovative test methodologies not usually used in the toxicological field. Cytokine

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secretion in human blood cells, toxic effects on hematopoietic system and several flow cytometric assays have been included in this WP. WP5 has developed alerts (based on physical and chemical properties) for those chemicals for which one or more processes may lead to a reduction of the actual or bioavailable concentration in the *in vitro* cytotoxicity assay. WP6 has developed a model for metabolism-dependent toxicity by comparing effects on metabolizing cells with non-metabolizing cells. Organ-specific toxicity has been studied by WP7 including several *in vitro* models for neurotoxicity, nephrotoxicity and liver toxicity. The results produced in the other WPs will be integrated into an *in vitro* test strategy by the WP8 which will be prevalidated during the last 2 years of the project (WP9). To follow the evolution and updating of the project, the website www.acutetox.org can be consulted. It is expected that further validation will lead to regulatory approval of the test strategy and its incorporation into standardized test guidelines for chemicals' hazard assessment.

As a part of the WP4, our group is responsible for the characterization of the hematotoxic effect of the chemicals. The hematopoietic system displays a wide spectrum of cell populations whose constant proliferation and differentiation in the bone marrow give rise to erythroid, granulocitic, macrophagic, megakaryocytic and lymphoid blood cells. In adults, hematopoietic progenitor cells are present mainly in the bone marrow although they can be also found in mobilized peripheral blood. It is an extremely complex tissue which is regulated by an equally complex set of hematopoietic growth factors by specific interactions with the stromal cells of the hematopoietic environment. Hematopoietic tissue is hierarchically organized; a small number of stem cells give rise to a whole variety of morphologically and functionally differentiated cells. The stem cell population is the fundamental base from which all the blood lines are derived. The main property of this population is its ability to maintain its own number. Committed progenitors are derived from the stem cells and are committed to a particular line of the hematopoietic development. These are transit cells that amplify the population prior to the production of mature cells (Fig. 1). Experimental procedures facilitating the formation of *in vitro* colonies were developed more than 40 years ago for studying the properties of the hematopoietic progenitors (Bradley and Metcalf, 1966). These techniques constituted powerful tools in the research of the hematopoietic system (Metcalf and Moore, 1971; Testa and Molineux, 1993). However, their application in toxicology, especially in humans, has not been so frequent, although there are several reports describing the sensitivity of hematopoietic progenitors to different xenobiotics (Okunewick and Marsh, 1985; OKunewick et al., 1990; Minderman et al., 1994). The availability of cord blood cells (CBC) as a rich source of human hematopoietic progenitors (Gluckman, 1995) facilitated to conduct hematotoxicity studies, once it was clarified that the sensitivity of cord blood progenitor cells was similar compared to their counterparts in the bone marrow (Leglise et al., 1996; Ghielmini et al., 1998; Gribaldo et al., 1999; Gómez et al., 2001).

The aim of this paper is to report the *in vitro* sensitivity of granulomonocytic progenitors to a number of chemicals included in the ACuteTox Project. The culture of human CFU-GM (Colony Forming Unit of Granulocytes and Monocytes) progenitors was used according to a protocol developed in a former project ("*In vitro* tests for haematotoxicity: prevalidation and validation of colony forming unit-granulocyte/macrophage (CFU-GM) assays for predicting acute neutropenia") supported by the European Center for the Validation of Alternative Methods (ECVAM) (Pessina et al., 2003). This is the first time that such a number of different chemicals have been tested in this *in vitro* system. Correlations of our results with human values are compared to the correlations of classical *in vitro* basal cytotoxicity tests such as 3T3 Neutral Red Uptake (NRU), normal human keratinocytes (NHK) NRU and HL-60 viability assays.

Methods

Human cells. Briefly, mononuclear cord blood cells (CBC) were obtained from umbilical cord blood scheduled for discard and processed during the next 12 h after birth. Cells were kept frozen and stored in liquid nitrogen. Prior using, CBC were thawed and diluted 1:1 with human albumin 2.5% (Behring, Hoechst Iberica,

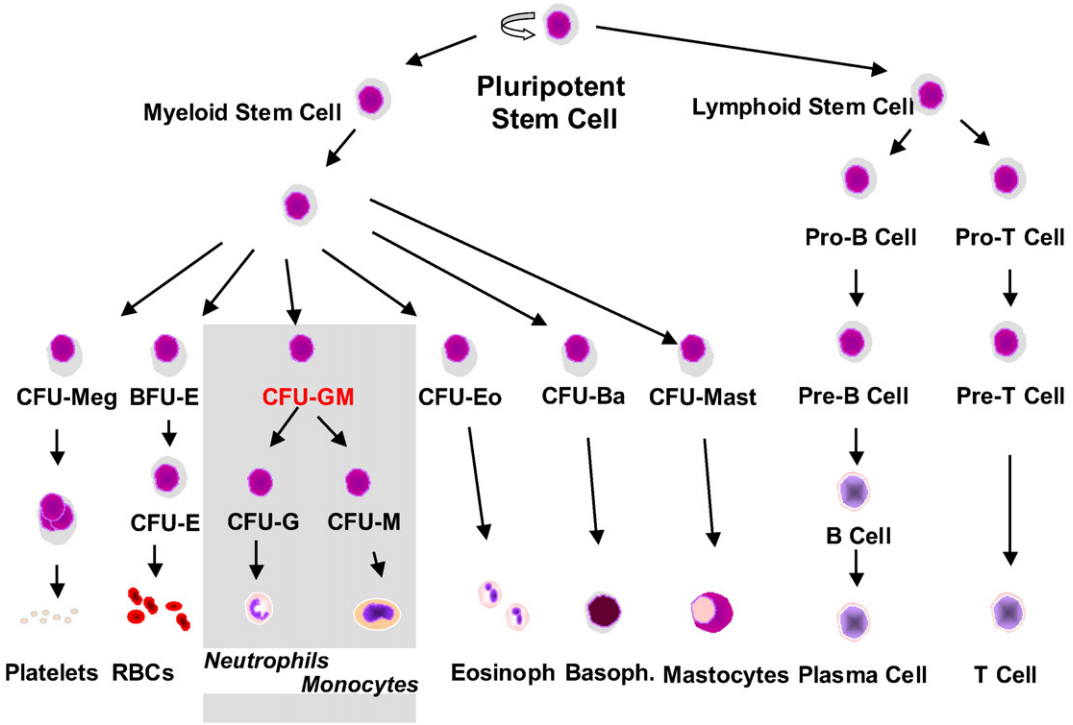

Fig. 1. Scheme of the hematopoietic system organization (Albella et al., 2007) highlighting the CFU-GM committed progenitors and their produced mature cells, granulocytes (neutrophils) and monocytes.

Spain) and 5% dextran 40 (Rheomacrodex 10%; Pharmacia Biotech, Uppsala, Sweden), and maintained at room temperature for 10 min. Cells were then diluted with IMDM (GIBCO, Grand Island NY) and centrifuged at 800 *g* for 15 min. After washing, cells were dispersed in IMDM supplemented with 10% FBS (Lonza, Walkersville, MD). At least three different donors were used for the testing of each chemical.

Clonogenic assays. According to the corresponding SOP, 50,000 mononuclear viable CBC were seeded in dishes containing MethoCult H4100 (StemCell Technologies, Vancouver, BC) in the presence of different doses of each chemical. Cells were plated into triplicate 35 mm culture dishes per condition (controls and cultures exposed to chemicals). The medium contained: 1% methylcellulose in IMDM (Iscove's Modified Dulbecco's Medium), 30% fetal bovine serum (FBS), 1% bovine serum albumin, 2 mM L-glutamine, and 10 ng/ml Granulocyte/Monocyte-Colony Stimulating Factor (human-rec-GM-CSF). Because of the presence of GM-CSF, this medium only supports the growth of committed myeloid progenitors, named CFU-GM (Colony Forming Unit of Granulocytes and Monocytes). The media had two basic conditions: a) the absence of 2-mercaptoethanol (which could protect the cells against toxicants; and b) the presence of cytokines free of carrier proteins to avoid drug concentration on the cells (Pessina et al., 2001). After 14 days of incubation at 37 °C in 5% CO_2 and fully humidified air, the CFU-GM colonies (Fig. 2) were scored under an inverted microscopy following a random fashion and standardized criteria (Pessina et al. 2001).

Chemicals. In all instances, a first set of experiment was designed in order to find the range of doses with effect. Whenever possible, data of human plasma levels from literature were used as indicative doses of exposures *in vitro*. When these data were not available, a half-log drop schema was used to find the range with effect. In a second set, doses were chosen within the IC30–IC90 range in order to fine-tune the dose–response curve between critical concentrations. When no growth inhibition was obtained, the chemicals were tested at the maximum concentration allowed taking into account the solubility and the concentration needed prior adding to the semisolid medium. The methylcellulose medium, the cells and the chemical to be tested were mixed immediately before putting onto the culture dishes. The different concentrations of each chemical were prepared from the stock solution by serial dilutions in sterile Eppendorf tubes at a concentration that was 100× the final dilution in the culture plate.

Statistical methods. All the results were calculated as survival percentages in respect to control cultures. Whenever DMSO was used as a solvent of the chemicals, the results were calculated as survival percentages in respect to control cultures exposed to 0.1% DMSO (final concentration of drug vehicle in cultures). The survival data were fitted by least squares only for experiments with at least three available data points. The IC50, IC70 and IC90 were obtained algebraically, solving the fitted quadratic equation for the value of dose where the estimated percentage of surviving cells would equal 50, 30 and 10% respectively (Albella et al., 2002; Gómez et al., 2003). Data in Table 1 show the mean ± SD and the coefficients of variation of at least three independent experiments.

Linear regression analysis was used to correlate IC50, IC70 and IC90 values with human LC50 (50% lethal concentration) values previously reported (Sjöström et al., 2008). Briefly, human blood concentration data, at acute poisoning, was collected from clinical and forensic medical case reports. Human LC50 were estimated using time-related human sublethal and lethal blood concentrations. These concentrations were plotted against time in hours after exposure and separate models were then fitted to the sublethal and lethal data. The regression model was then extrapolated to time 0. Then, the arithmetical mean of sublethal and lethal doses extrapolated at time 0 was used as an estimate of the human blood LC50 value for a chemical (Sjöström et al., 2008).

Results

Eleven out of 55 compounds did not show any effect at the maximum concentration tested, so they were not used in the *in vitro-in vivo* correlation. For the rest of the chemicals, IC50, IC70 and IC90 values were calculated (Table 1). Despite the high variability that is

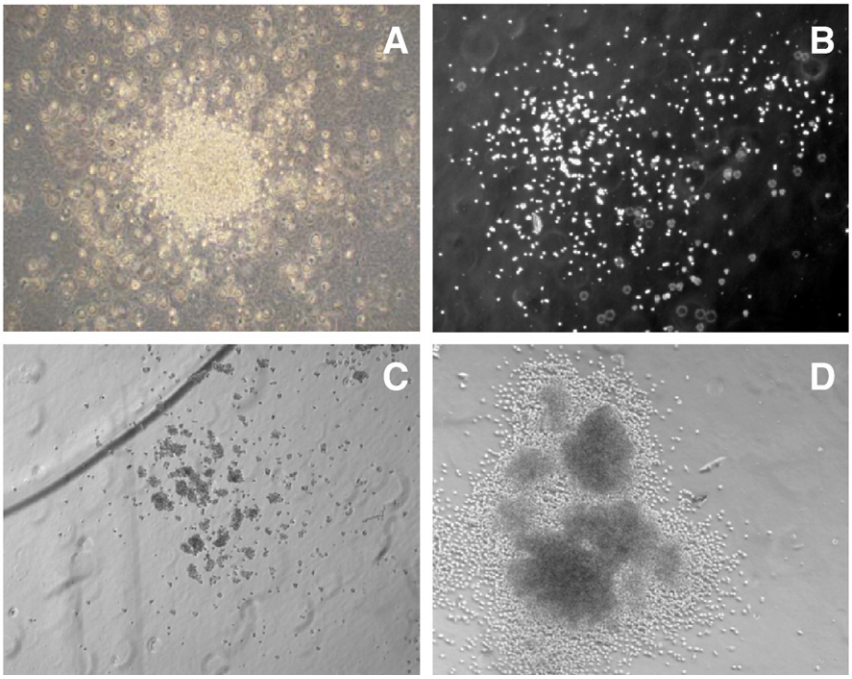

Fig. 2. Microphotographs of different types of CFU-GM colonies. (A) Compact colony: with a central dense nucleus and a peripheral halo. (B) Diffuse and spread colony without apparent nucleus. (C) Burst-forming-like colony: multifocal colony with several aggregates, with of without a peripheral halo. (D) Multicentric colony with two or more dense nuclei nearby, with a common peripheral halo growing at the same depth in the plate.

Table 1
IC50, IC70 and IC90 values for the chemicals tested in CFU-GM assay.

Number in Acutoxbase	Reference chemical	IC50 ± SD (M) CV (%)	IC70 ± SD (M) CV (%)	IC90 ± SD (M) VC CV (%)
1	Acetaminophen	2.06E-4 ± 4.49E-5 (21.79)	2.86E-4 ± 4.36E-5 (15.24)	3.66E-4 ± 5.08E-5 (13.88)
2	Acetylsalicylic acid	9.83E-4 ± 6.82E-5 (6.93)	1.37E-3 ± 1.42E-4 (10.29)	1.77E-3 ± 2.89E-4 (16.37)
3	Atropine sulfate monohydrate	2.71E-4 ± 5.34E-5 (19.79)	4.14E-4 ± 2.24E-5 (5.41)	5.57E-4 ± 4.65E-5 (8.36)
4	Caffeine	5.93E-4 ± 5.51E-5 (9.29)	8.87E-4 ± 1.27E-5 (1.43)	1.17E-3 ± 2.97E-5 (2.52)
5	Carbamazepine	No effect at maximum tested dose (6.40E-5)		
6	Colchicine	7.74E-9 ± 8.03E-10 (10.37)	1.09E-8 ± 1.23E-9 (11.25)	1.38E-8 ± 1.67E-9 (12.13)
7	Cyclohexamide	5.05E-7 ± 1.99E-7 (39.39)	7.24E-7 ± 1.63E-7 (22.46)	9.42E-7 ± 1.32E-7 (14.02)
8	Diazepam	2,53E-4 ± 1.61E-5 (22.79)	3.85E-4 ± 6.53E-5 (23.98)	5.17E-4 ± 1.14E-4 (31.28)
9	Digoxin	5.52E-8 ± 6.43E-9 (15.60)	6.43E-8 ± 1.18E-8 (18.41)	7.34E-8 ± 1.44E-8 (19.60)
10	Isopropyl alcohol	No effect at maximum tested dose (1.30E-1)		
11	Malathion	7.13E-4 ± 1.99E-4 (24.90)	9.58E-4 ± 2.34E-4 (24.37)	1.20E-3 ± 2.99E-4 (24.80)
12	Mercury(II) chloride	8.64E-06 ± 6.73E-7 (7.79)	1.35E-5 ± 2.05E-6 (15.18)	1.84E-5 ± 4.13E-6 (22.42)
13	Pentachlorophenol	No effect at maximum tested dose (2.00E-4)		
14	Phenobarbital	No effect at maximum tested dose (8.00E-4)		
15	Sodium lauryl sulfate	6.03E-4 ± 1.20E-4 (19.89)	8.05E-4 ± 1.73E-4 (21.44)	1.01E-3 ± 2.32E-4 (23.06)
16	Sodium valproate	1.34E-3 ± 1.27E-4 (9.48)	2.02E-3 ± 1.96E-4 (9.70)	2.70E-3 ± 2.66E-4 (9.87)
17	5-fluorouracil	3.80E-6 ± 1.33E-6 (24.85)	5.61E-6 ± 1.53E-6 (24.17)	7.43E-6 ± 1.73E-6 (23.27)
18	Benzene	No effect at maximum tested dose (1.12E-1)		
19	*tert*-Butylhydroperoxide	2,83E-5 ± 1.31E-6 (4.63)	3.79E-5 ± 5.51E-6 (14.53)	4.76E-5 ± 9.72E-6 (20.42)
20	Acrylaldehyde (acrolein)	1.61E-5 ± 3.89E-6 (24.1)	2.38E-5 ± 5.69E-6 (23.87)	3.15E-5 ± 7.49E-6 (23.77)
21	Cadmium(II) chloride	1.20E-4 ± 5.79E-5 (30.66)	1.66E-4 ± 4.04E-5 (21.84)	2.09E-4 ± 2.98E-5 (18.08)
23	Pyrene	No effect at maximum tested dose (1.00E-4)		
26	Hexachlorobenzene	6.00E-5 ± 1.21E-5 (20.14)	8.39E-5 ± 1.15E-5 (13.71)	1.08E-4 ± 1.26E-5 (11.64)
28	Amiodarone hydrochloride	2.10E-5 ± 1.27E-5 (60.57)	3.16E-5 ± 9.68E-6 (30.76)	4.22E-5 ± 7.36E-6 (17.53)
29	Verapamil hydrochloride	1.33E-5 ± 7.92E-6 (59.51)	2.03E-5 ± 7.52E-6 (37.19)	2.71E-5 ± 7.37E-6 (24.01)
30	Rifampicine	4.12E-5 ± 7.41E-6 (18.07)	6.62E-5 ± 7.43E-6 (11.17)	9.13E-5 ± 1.12E-5 (12.28)
31	Tetracycline hydrochloride	5.55E-5 ± 3.31E-5 (59.64)	1.03E-4 ± 4.22E-5 (40.99)	1.50E-4 ± 5.15E-5 (34.02)
32	Orphenadrine hydrochloride	6.41E-5 ± 1.68E-5 (24.24)	9.31E-5 ± 1.42E-5 (15.30)	1.22E-4 ± 1.24E-5 (10.14)
33	Nicotine	2.28E-3 ± 1.46E-4 (6.38)	2.70E-3 ± 3.34E-4 (12.35)	3.10E-3 ± 5.24E-4 (16.86)
34	Lindane	5.07E-5 ± 6.20E-6 (12.24)	7.05E-5 ± 4.92E-6 (6.98)	9.04E-5 ± 9.04E-5 (7.53)
37	Ethanol	No effect at maximum tested dose (1.70E-1)		
39	Dichlorvos	1.13E-5 ± 1.24E-6 (15.57)	1.73E-5 ± 2.53E-6 (20.66)	2.33E-5 ± 3.81E-6 (23.07)
40	Physostigmine	2.62E-5 ± 3.08E-6 (11.74)	3.23E-5 ± 2.08E-6 (6.46)	3.83E-5 ± 1.60E-6 (4.17)
41	Glufosinate ammonium	3.10E-3 ± 3.02E-3 (97.32)	5.52E-3 ± 2.45E-3 (44.37)	8.09E-3 ± 1.93E-3 (23.87)
42	*cis*-Platinum	2.28E-4 ± 1.01E-5 (4.45)	3.14E-4 ± 3.67E-5 (11.60)	4.00E-4 ± 6.41E-5 (16.01)
43	Diethylene glycol	7.60E-2 ± 5.60E-3 (7.40)	1.01E-1 ± 1.29E-2 (12.76)	1.26E-1 ± 2.03E-2 (15.87)
45	Ochratoxin A	3.57E-5 ± 5.45E-6 (15.27)	5.46E-5 ± 1.08E-5 (19.83)	7.35E-5 ± 1.63E-5 (22.12)
47	Ethinyl estradiol	4.28E-5 ± 5.73E-6 (13.44)	5.73E-5 ± 6.53E-6 (11.41)	7.18E-5 ± 9.35E-6 (13.02)
48	Sodium fluoride	1.21E-3 ± 1.74E-4 (14.44)	1.56E-3 ± 1.52E-4 (9.74)	1.92E-3 ± 1.67E-4 (8.69)
51	Dimethylformamide	4.65E-2 ± 2.94E-3 (6.26)	7.10E-2 ± 1.09E-2 (15.30)	9.48E-2 ± 1.78E-2 (18.93)
53	Amitrypline hydrochloride	2.39E-5 ± 4.34E-6 (18.16)	3.22E-5 ± 3.71E-6 (11.45)	4.06E-5 ± 3.08E-6 (7.58)
54	Ethylene glycol	No effect at maximum tested dose (1.80E-1)		
55	Methanol	No effect at maximum tested dose (2.47E-1)		
57	Sodium chloride	No effect at maximum tested dose (4.00E-2)		
60	Litium sulphate	4.46E-3 ± 1.28E-3 (24.61)	6.04E-3 ± 1.26E-3 (20.88)	7.61E-3 ± 1.37E-3 (18.04)
63	Propanolol hydrochloride	4.29E-5 ± 7.91E-6 (18.39)	5.81E-5 ± 8.97E-6 (15.42)	7.33E-5 ± 1.05E-5 (14.37)
64	Arsenic trioxide	9.40E-6 ± 5.41E-6 (57.53)	1.35E-5 ± 6.75E-6 (49.87)	1.77E-5 ± 8.45E-6 (47.82)
66	Thallium sulphate	1.49E-5 ± 4.57E-6 (24.55)	2.22E-5 ± 3.35E-6 (15.09)	2.96E-5 ± 2.21E-6 (7.53)
67	Warfarin	3.00E-4 ± 4.89E-5 (16.40)	3.95E-4 ± 7.81E-5 (19.73)	4.90E-4 ± 1.12E-4 (22.76)
76	Chloral hydrate	4.61E-1 ± 2.61E-2 (8.00)	6.35E-1 ± 7.47E-2 (16.64)	8.08E-1 ± 1.23E-1 (21.57)
77	2,4-Dichlorophenoxyacetic acid	8.69E-4 ± 2.25E-4 (24.98)	1.41E-3 ± 3.26E-4 (23.12)	1.95E-3 ± 4.85E-4 (24.82)
80	Strychnine	4.55E-5 ± 5.18E-6 (11.38)	5.97E-5 ± 5.97E-6 (10.00)	7,39E-5 ± 6.82E-6 (9.22)
91	Sodium selenate	1.12E-4 ± 1.68E-5 (14.94)	1.32E-4 ± 1.61E-5 (12.15)	1.53E-4 ± 1.54E-5 (10.10)
92	Acetonitrile	No effect at maximum tested dose (1.90E-1)		
96	Epinephrine bitartrate	3.28E-5 ± 3.01E-6 (9.15)	4.50E-5 ± 2.96E-6 (6.58)	5.72E-5 ± 3.94E-6 (6.87)

CV, coefficients of variation (%).

intrinsic to the use of primary human cells, within-laboratory reproducibility of experimental data obtained was very good: 89% of the IC values showed coefficients of variation (CV) below 25% (Table 1).

IC values were compared to the human LC50 values estimated by the model described by WP3 (Sjöström et al., 2008). The LC50 values were calculated from time-related sublethal and lethal blood concentrations determined from human acute poisoning cases. Only chemicals, for which both *in vivo* and *in vitro* data were available, were used for the correlations with human LC50 values. A total of 43 out of 55 compounds studied on human myeloid hematopoietic progenitors (CFU-GM) could be used in the correlations. Besides the exclusion of the compounds that did not show effect (chemicals 5, 10, 13, 14, 18, 23, 37, 54, 55, 57 and 92), chloral hydrate was also excluded because the *in vivo* human available data was from its metabolite, trichloroethanol. Tert-butyl-hydroperoxide, cadmium II chloride, benzene and 5-FU were included in our WP as positive controls because they have been previously proved to exert *in vivo* effect on the hematopoietic system (Grem, 1996; Yoon et al., 2001). As it was expected, most of these drugs showed inhibition of colony growth in the range of micromolar concentration. Only benzene failed to affect the *in vitro* formation of CFU-GM colonies. The *in vitro-in vivo* model between IC50 values (x) from the CFU-GM assay and human LC50 values (y) gave a regression model of $y = 0.7774x - 1.2602$ and with $R^2 = 0.4965$. When IC70 and IC90 values were used, although the differences were not statistically significant, the R^2 increased to 0.5106 and 0.5142 respectively (Fig. 3).

Our results have also been compared to the IC values obtained with two more assays also validated, included in the ACuteTox Project and recently reported by Clothier et al. (2008). These are the Neutral Red

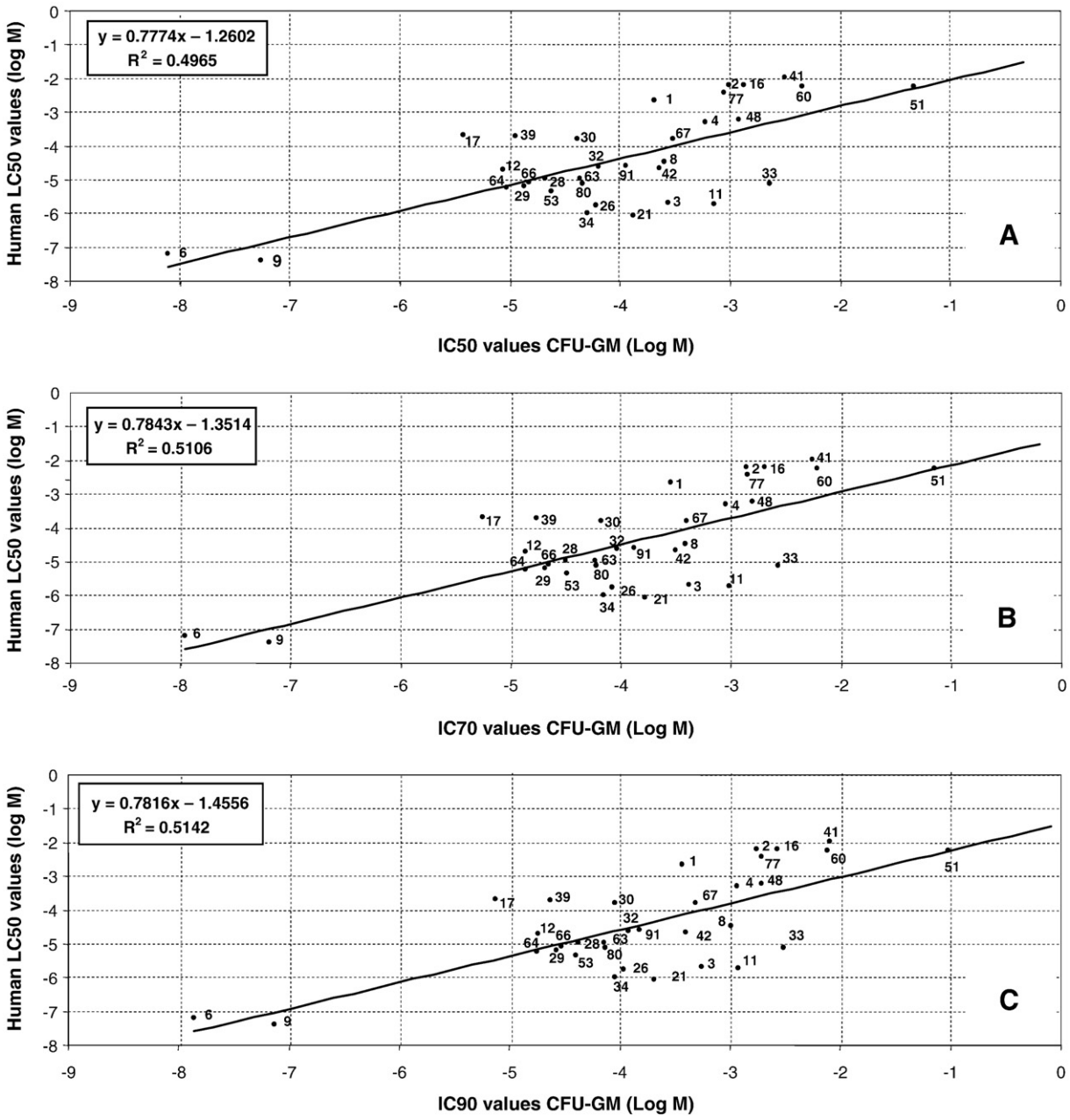

Fig. 3. Plot of human LC50 values (log M) estimated in the ACuteTox Project (Sjöström et al., 2008) versus human IC50 (A), IC70 (B) and IC90 (C) values (log M) estimated in the CFU-GM assay.

Uptake (NRU) determined in the 3T3 cell line and normal human keratinocytes (NHK) (Spielmann et al., 2008). The results obtained in these studies demonstrated the usefulness of human LC50 values obtained in basal cytotoxicity assays (3T3 NRU) for human acute systemic toxicity.

Most of the IC50 values from the CFU-GM assay were lower compared to values from the 3T3 NRU assay, showing a general higher sensitivity of human CFU-GM progenitors (Table 2). Furthermore, 33% of the IC50 values from the CFU-GM assay showed at least 1 log difference compared to values from 3T3 NRU assay. Linear regression analysis between IC50 and LC50 values gave a correlation coefficient (R^2) of 0.56 for 3T3 NRU (Sjöström et al., 2008) and 0.59 for NHK NRU (Fig. 4A). When we studied the correlation between IC50 values of CFU-GM assay and the IC50 values of 3T3 and NHK cells, the linear regression analysis gave R^2 values of 0.54 and 0.61 respectively (Figs. 4B and C).

Since the HL-60 cell line is used in toxicological studies as a model to obtain hints of effect of xenobiotics on the hematopoietic tissue, we have also performed linear regression analysis between HL-60 IC50 values by means of luminescence ATP assay (Clothier et al., 2008) with respect to the LD50 data (Fig. 5). It can be firstly observed that CFU-GM progenitors were more sensitive than this cell line; 46% of the compounds showed IC50 values 1 log lower in CFU-GM (Table 2). The linear regression analysis between HL-60 IC50 and human LC50 values gave a correlation coefficient of 0.39 and there was no correlation between IC50 data from CFU-GM and HL-60 cells ($R^2 = 0.14$).

Discussion

Cultures of human CFU-GM progenitors were used as described in a protocol developed in a former project ("*In vitro* tests for haematotoxicity: prevalidation and validation of colony forming

Table 2
IC50 values (log M) from CFU-GM, HL-60 ATP assay, 3T3 NRU assays and human LC50 values (log M).

N° in Acutoxbase	Reference chemical	CFU-GM IC50 (log M)	HL-60 IC50[a] (log M)	3T3 IC50[b] (log M)	NHK IC50[a] (log M)	Human LC50[b] (log M)
1	Acetaminophen	−3.69	−2.08	−3.48	−2.46	−2.65
2	Acetylsalicylic acid	−3.01	−2.21	−2.36	−2.46	−2.20
3	Atropine sulfate monohydrate	−3.57	−2.78	−3.91	−3.88	−5.70
4	Caffeine	−3.23	−2,00	−3.08	−2.47	−3.29
5	Carbamazepine	>−4.19	−3.11	ND	−3.27	ND
6	Colchicine	−8.11	−2.06	−5.98	−7.76	−7.19
7	Cyclohexamide	−6.30	ND	−2.78	−6.51	ND
8	Diazepam	−3.60	−3.52	−3.18	−3.96	−4.49
9	Digoxin	−7.26	>−2.97	−2.88	−8.38	−7.38
10	Isopropyl alcohol	>−0.89	−0.87	ND	−1.24	ND
11	Malathion	−3.15	−3.17	−2.88	−3.40	−5.73
12	Mercury(II) chloride	−5.06	−4.79	−4.80	−4.66	−4.71
13	Pentachlorophenol	>3.69	−3.88	ND	−5.26	−3.08
14	Phenobarbital	>−3.09	−2.08	ND	−2.69	−3.4
15	Sodium lauryl sulfate	−3.22	−3.57	−3.79	−4.96	ND
16	Sodium valproate	−2.87	−1.97	−2.00	−2.25	−2.20
17	5-fluorouracil	−5.42	−2.98	−4.30	−3.00	−3.69
18	Benzene	>−0.95	−1.28	ND	−0.78	ND
19	*tert*-Butylhydroperoxide	−4.55	−4.46	−4.21	−3.59	ND
20	Acrylaldehyde (acrolein)	−4.79	−4.12	−3.07	−4.58	ND
21	Cadmium(II) chloride	−3.88	−4.32	−5.65	−4.97	−6.06
23	Pyrene	>−4.00	>2.60	ND	−3.83	ND
26	Hexachlorobenzene	−4.22	>−4.45	>−3.40	ND	−5.77
28	Amiodarone hydrochloride	−4.68	−4.53	−4.58	−5.44	−4.95
29	Verapamil hydrochloride	−4.88	−3.57	−4.14	−3.85	−5.21
30	Rifampicine	−4.39	−3.75	−3.99	−3.76	−3.81
31	Tetracycline hydrochloride	−4.26	−3.46	−3.08	−3.56	ND
32	Orphenadrine hydrochloride	−4.19	−3.47	−3.95	−3.68	−4.64
33	Nicotine	−2.64	−2.34	−2.49	−2.71	−5.10
34	Lindane	−4.29	−3.85	−3.27	−4.18	−5.98
37	Ethanol	>−0.77	−0.26	ND	−0.64	−0.80
39	Dichlorvos	−4.95	ND	−3.77	−4.29	−3.70
40	Physostigmine	−4.58	ND	−3.99	ND	ND
41	Glufosinate ammonium	−2.51	−1.67	−2.12	−3.19	−1.99
42	*cis*-Platinum	−3.64	−3.17	−5.25	−4.79	−4.68
43	Diethylene glycol	−1.12	−0.55	−0.89	−0.63	ND
45	Ochratoxin A	−4.45	−4.28	−4.18	−6.87	ND
47	Ethinyl estradiol	−4.37	−4.44	−4.64	−6.87	ND
48	Sodium fluoride	−2.92	ND	−2.72	−2.92	−3.24
51	Dimethylformamide	−1.33	ND	−1.14	−0.97	−2.23
53	Amitrypline hydrochloride	−4.62	ND	−4.64	−4.51	−5.34
54	Ethylene glycol	>−0.74	ND	ND	−0.23	−1.50
55	Methanol	>−0.61	ND	ND	−1.32	−1.11
57	Sodium chloride	>−1.39	ND	ND	−0.96	−1.27
60	Litium sulphate	−2.35	ND	−1.81	ND	−2.25
63	Propanolol hydrochloride	−4.37	ND	−4.27	−3.96	−4.95
64	Arsenic trioxide	−5.03	ND	−4.87	−4.31	−5.23
66	Thallium sulphate	−4.83	ND	−4.80	−6.50	−5.09
67	Warfarin	−3.52	ND	−3.09	−3.41	−3.81
76	Chloral hydrate	−0.34	ND	−1.81	−3.08	−3.18
77	2,4-Dichlorophenoxyacetic acid	−3.06	ND	−4.27	ND	−2.43
80	Strychnine	−4.34	ND	−4.87	−3.65	−5.12
91	Sodium selenate	−3.95	ND	−4.80	−4.23	−4.59
92	Acetonitrile	>−0.72	ND	ND	−0.63	−2.82
96	Epinephrine bitartrate	−4.48	ND	−3.09	−3.57	ND

ND: not determined.
[a] The IC50 values for HL-60 assay are available from Clothier et al. (2008).
[b] The IC50 values for 3T3 NRU assay and the human LC50 values are available from Sjöström et al. (2008).

unit-granulocyte/macrophage (CFU-GM) assays for predicting acute neutropenia") supported by ECVAM (Pessina et al., 2003). This assay has been extensively used for predicting the hematotoxicity of antitumoral compounds (Albella et al., 2007). However, to our knowledge, this is the first time that so many and different chemicals have been tested on primary human hematopoietic cells with this already validated *in vitro* culture. From the studies with antitumoral compounds, it is known that the drug exposure level that inhibits CFU-GM by 90% *in vitro* and decreases neutrophils by 90% *in vivo* were the same (Parchment et al., 1994). This reduction *in vivo* corresponds to grade 4 neutropenia, which is considered dose-limiting (acute) toxicity. The importance of the IC90 value in the hematopoietic field was definitely confirmed with the results of the validation of the CFU-GM assay (Pessina et al., 2001, 2002, 2003, 2005). The prediction model is therefore highly dependent upon using the correct degree of inhibition. Because of that, in this study, together with the IC50 value, the IC70 and the IC90 values have also been calculated. Although the *in vitro-in vivo* correlation is not improved in the CFU-GM assay (R^2 0.51) in respect to NRU 3T3 assay (R^2 0.56), the correlation with human LC50 values tends to increase when IC70 or IC90 data are used compared to IC50 values. It is likely that the inclusion of compounds with a higher toxicity, such as antitumorals, would give more accuracy to the correlation by introducing more data in the range of micro and nanomolar. In order to use the data from CFU-GM cultures as an organ-specific assay of the hematopoietic myeloid system, our results suggest that it would be better to use IC90 rather than IC50 values.

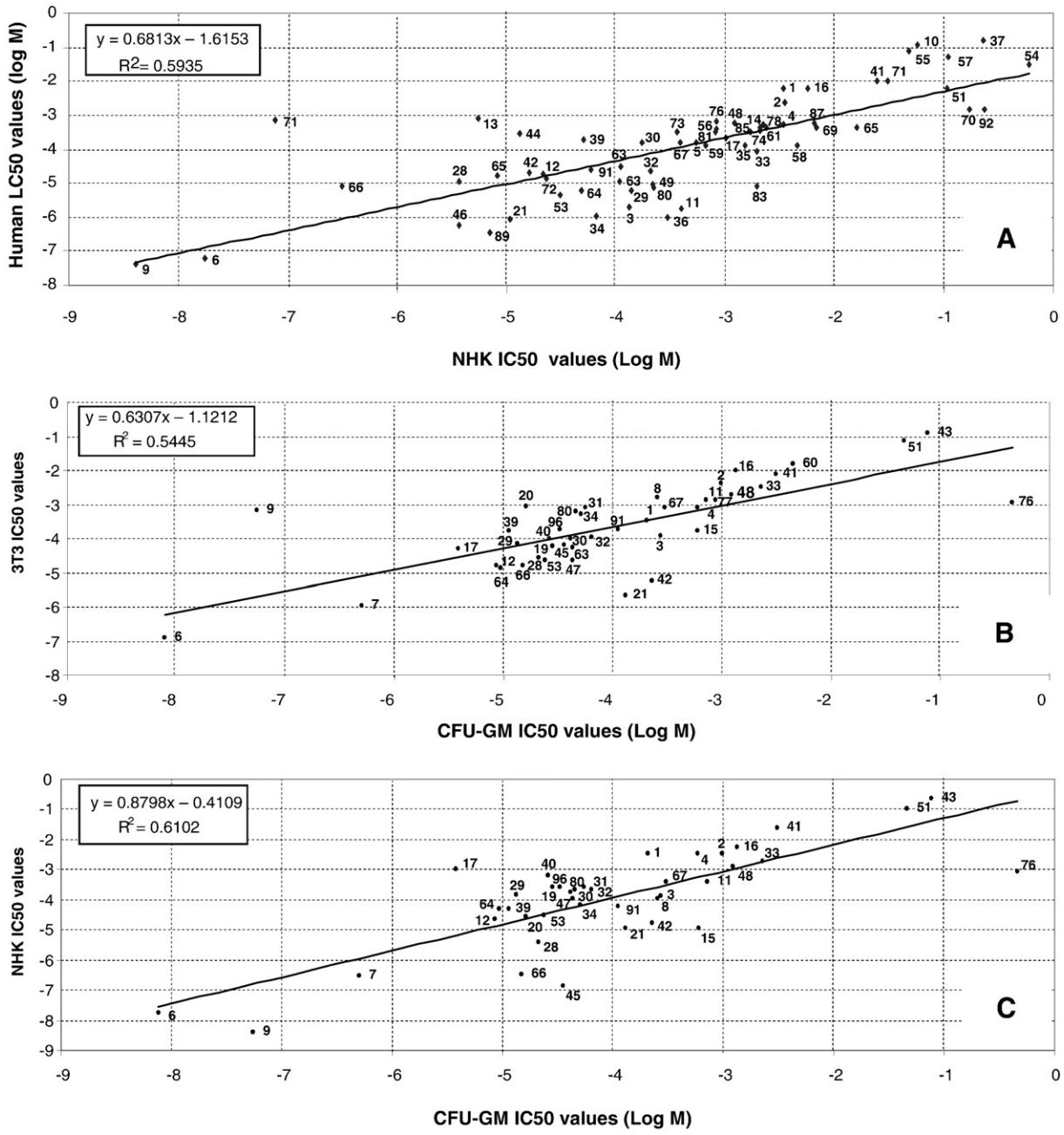

Fig. 4. Plot of human LC50 values (log M) estimated in the ACuteTox Project (Sjöström et al., 2008) versus NHK IC50 values (A), and CFU-GM IC50 values versus IC50 values from 3T3 (B) and NHK (C) cells.

Although correlations of CFU-GM values with human LC50 data did not significantly improve correlations obtained in other *in vitro* cytotoxicity tests, as the 3T3 and NHK NRU assays, some facts are worthy of being mentioned. Also in the context of ACuteTox Project, Clothier et al. (2008) reported the IC50 values obtained from six basal cytotoxicity assays: 3T3, NHK and Fa32 cells with NRU assay, HepG2 and Fa32 cells with the CBQCA (3-(4-carboxybenzoyl)quinoline-2-carboxaldehyde) total protein assay, and HL-60 cells with the ATP endpoint assay. When the six assays were simultaneously correlated to human LD50 values, a high R^2 correlation (0.83) was obtained, revealing that the majority of the chemicals behaved similarly. However, for each individual cytotoxicity test there were, for all the tests, a few chemicals deviated substantially by more than one order of magnitude and a number of chemicals showed a high variability among the tests. Curiously, some of them, colchicine, digoxin, 5-Fluorouracil and thallium sulfate showed good correlation in the CFU-GM assay.

Taking into account the high proliferative rate of the committed hematopoietic progenitors, it was not unexpected that chemical compounds, whose mechanism of action has to do with the inhibition of cell cycle progression (i.e. 5-FU, colchicine), highly affect the human CFU-GM progenitors. However, it was surprising to find *in vitro* effect of drugs affecting cells through other specific mechanisms of action such as diazepam or digoxin, although it is not the first time that such phenomenon is described. Some atypical antipsychotics may lead to agranulocytosis and clozapine's analogues have been reported as causing neutropenia. Clozapine, olanzapine, quetiapine, and chlorpromazine were characterized by dose-dependent toxicity on CFU-GM progenitors (Pessina et al., 2006).

On the other hand, we also compared our results with the ones obtained from HL-60 cells (Clothier et al., 2008). This cell line is usually used in toxicological and preclinical studies to obtain data on the effect of xenobiotics on hematopoietic tissue. Besides being a cell

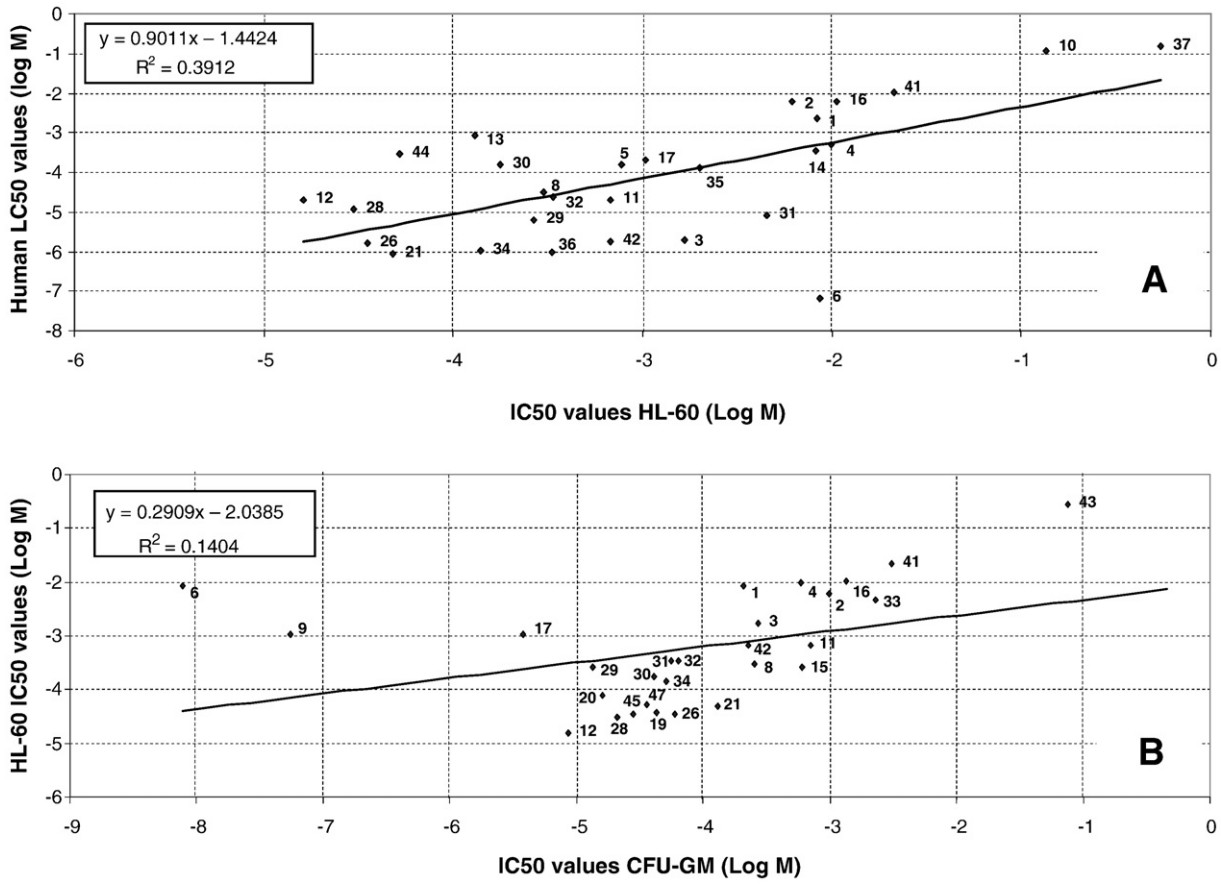

Fig. 5. Plot of human LC50 values (log M) estimated in the ACuteTox Project (Clothier et al., 2008) versus HL-60 IC50 values (A), and CFU-GM IC50 values versus IC50 values from HL-60 cells (B).

line, HL-60 cells are leukemic and possess characteristics which may conduct to a different sensitivity to xenobiotics. In the ACuteTox Project, data from both leukemic (HL-60 cells) and normal (CFU-GM) hematopoietic cells have been obtained. Leukemic cells have been studied by means of detecting cell viability through an ATP luminescence assay. The results showed that, for the 46% of the chemicals, CFU-GM progenitors were one order of magnitude more sensitive than HL-60 cells, which highlights the inconvenience of coming to conclusions after using data obtained from non normal hematopoietic cells. Moreover, it is worthy to mention that we found that there is no correlation between IC50 values of these leukemic cells and normal CFU-GM progenitors.

Tert-butyl-hydroperoxide, cadmium II chloride, benzene and 5-FU were included in our WP as positive controls of effect on hematopoietic system (Grem, 1996; Yoon et al., 2001). The last of them was also tested in the previous validation process of the CFU-GM assay (Pessina et al., 2003), showing an IC50 value of 5.4×10^{-6} M, which is not significantly different from the one obtained in the ACuteTox Project (3.8×10^{-6} M). All control compounds showed an effect on the myeloid progenitor except for benzene, such as was previously reported in *in vitro* assays. This compound is hematotoxic when administered *in vivo* because of their metabolites (Van Den Heuvel et al., 2001). Although a number of studies suggests that hematopoietic cells themselves could bioactivate some compounds, this capacity must be very low compared to total hepatic cell population (Pessina et al., 2006; Shao et al., 2007; Croera et al., 2008; Ferrario et al., 2008). Therefore, CFU-GM assay could fail in predicting the toxicity of compounds that must be metabolized priorly to exert toxicity. Some attempt has been done with the aim to assess the susceptibility of CFU-GM in the presence of a metabolic system, however it did not fully succeed (Dal Negro et al., 2006). This limitation could be avoided by complementing with the *in vitro* assays developed in WP5 and WP6 (biokinetics and biotransformation) of the ACuteTox Project. Another drawback of the CFU-GM assay is that it may be considered a time-consuming test and, in its current design, as performed in the ACuteTox Project, it would be unsuitable for high throughput screening of xenobiotics. Although there are studies focusing on the redesign of this assay into a more rapid one (Rich and Hall, 2005, Dal Negro et al., 2006; Pessina et al., 2006) any new strategy should be properly validated prior to application (Balls, 1995b, 1995a) because there are many experimental factors which could affect the effects of xenobiotics on these cells (Gribaldo et al., 1996).

In summary, the following conclusions arise from our results: a) CFU-GM assays based on the culture of human primary hematopoietic cells do not improve the correlation with human LD50 values, compared to the correlation obtained with the 3T3 NRU basal cytotoxicity assay, b) the sensitivity of CFU-GM progenitors is a better indication of hematotoxicity than the HL-60 cell line and, c) CFU-GM assays could be very helpful in refining the characterization of acute toxicity of chemicals as an alert of toxicity on the hematopoietic myeloid system. Once all the data produced in the ACuteTox Project are analyzed in an integrated way, the possible advantages of including this assay as a test for organ-specific toxicity (hematotoxicity) will finally be concluded.

Acknowledgments

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Rescue of Pyruvate Kinase Deficiency in Mice by Gene Therapy Using the Human Isoenzyme

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Human erythrocyte R-type pyruvate kinase deficiency (PKD) is a disorder caused by mutations in the *PKLR* gene that produces chronic nonspherocytic hemolytic anemia. Besides periodic blood transfusion and splenectomy, severe cases require bone marrow (BM) transplant, which makes this disease a good candidate for gene therapy. Here, the normal human R-type pyruvate kinase (hRPK) complementary (cDNA) was expressed in hematopoietic stem cells (HSCs) derived from *pklr* deficient mice, using a retroviral vector system. These mice show a similar red blood cell phenotype to that observed in human PKD. Transduced HSCs were transplanted into myeloablated adult PKD mice or *in utero* injected into nonconditioned PKD fetuses. In the myeloablated recipients, the hematological manifestations of PKD were completely resolved and normal percentages of late erythroid progenitors, reticulocyte and erythrocyte counts, hemoglobin levels and erythrocyte biochemistry were restored. Corrected cells preserved their rescuing capacity after secondary and tertiary transplant. When corrected cells were *in utero* transplanted, partial correction of the erythrocyte disease was obtained, although a very low number of corrected cells became engrafted, suggesting a different efficiency of cell therapy applied *in utero*. Our data suggest that transduction of human RPK cDNA in *PKLR* mutated HSCs could be an effective strategy in severe cases of PKD.

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INTRODUCTION

Pyruvate kinase (PK, EC 2.7.1.40) is a metabolic enzyme that catalyses the last step of glycolysis. PK transfers the phosphate group from phosphoenolpyruvate to adenosine diphosphate, generating pyruvate and adenosine triphosphate (ATP). Given the critical nature of this reaction, any loss in PK activity impairs cell metabolism.[1] In humans, four PK isoforms are expressed by two structural genes: The *PKLR* gene (PK liver and red blood cells gene) on Chr1q21, encodes the RPK (R-type pyruvate kinase) and L-type pyruvate kinase tissue specific isoforms expressed in erythroid cells and liver, respectively; and the *PKM* gene on Chr15q22 codes for isoforms M1-type pyruvate kinase, expressed in brain and skeletal muscle, and M2-type pyruvate kinase, expressed in fetal and most adult tissues except erythroid cells.[2] The expression of *PKLR*- derived isoenzymes is regulated by tissue specific promoters, whereas the two products of the *PKM* gene are synthesized by alternative mRNA splicing.[3]

Mutations in the *PKLR* gene[1,3,4] lead to pyruvate kinase deficiency (PKD), an autosomal recessive disorder, which is the most frequently enzymatic defect of the Embden-Meyerhof pathway in erythrocytes. Together with glucose-6-phosphate dehydrogenase deficiency, this is the most common hereditary enzymopathy causing chronic nonspherocytic hemolytic anemia.[5] Over 100 different mutations have been identified on the structural *PKLR* gene.[6] In most cases, they are missense mutations, although nonsense mutations, deletions, insertions, and even disruption of the erythroid promoter causing severe deficiency have been reported.[6–10] Generally, most patients are compound heterozygous with two different mutant alleles, but homozygous mutations with highly deleterious alleles causing life-threatening anemia have also been described.[6,10]

Although the global incidence of PKD is unknown, it has been estimated at 51 cases per million in North America.[11] Clinical symptoms of PKD vary considerably from mild to severe anemia. Pathological manifestations are usually observed when enzyme activity falls <25% normal PK activity, and severe disease has been associated with a high degree of reticulocytosis.[10,12] Currently, there is no definitive treatment for severe PKD (see review in ref. 13) as the *PKLR* gene is not functionally compensated in the erythrocyte by *PKM* isoenzymes.[10] Although splenectomy can be clinically useful in patients with severe disease, in some cases, allogeneic hematopoietic transplantation is required.[14] In these patients, hematopoietic stem cell (HSC) gene therapy might be a good and more effective treatment.

Animal models of PKD have been developed in dogs and mice.[15–17] In a dog model, bone marrow (BM) transplant without previous recipient conditioning failed to resolve hematological

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symptoms.[15] In mice, attempts have been made to selectively expand normal donor erythrocytes in minimally conditioned recipients.[18] Attempts to rescue RPK-deficient mice have been addressed using mouse transgenic cells that expressing the human RPK complementary (cDNA).[19] Human L-type pyruvate kinase has been expressed into murine HSC demonstrating long-term expression (3 months) of the human L-type pyruvate kinase protein in peripheral blood.[20] Recently we reported that transduction of BM cells using γ-retroviral vectors[21] carrying human RPK cDNA[22] mediates long-term expression of the human RPK protein in red blood cells obtained from primary and secondary recipients, without any detectable adverse effects.[23]

Additionally, as PKD is an inherited disease that can be diagnosed before birth,[24] the *in utero* transplant of genetically corrected HSC might be a treatment option, as proposed for other inherited diseases.[25] We have recently demonstrated in a mouse model, which genetically modified syngenic hematopoietic cells show efficient engraftment *in utero* and give rise to mature hematopoietic cells in adulthood,[26] thus providing phenotype correction from early development to adulthood. This approach avoids the problems associated to allogeneic transplantation, such us the need of a compatible donor and graft rejection.

In the present work, we show that human RPK expressing vectors are able to fully reverse the hemolytic phenotype in PKD mice. HSCs from these animals can be genetically corrected and transplanted into lethally irradiated adult PKD mice. In addition, our data indicate that the *in utero* transplant of these genetically corrected cells into nonconditioned PKD fetuses achieves partial correction of the disease.

RESULTS

The anemic phenotype is corrected in adult RPK-deficient mice by the transplantation of genetically corrected HSCs

To assess the efficacy of γ-retroviral vectors carrying human RPK cDNA to treat PKD mice, lineage negative (Lin^-) cells from the BM of PKD male mice were transduced with SF11XEG or SF11RPKXEG vectors (see Materials and Methods). At the end of the transduction protocol, transduced cells (2×10^5 cells/mouse) were transplanted into lethally irradiated female PKD mice. Erythroid variables were determined in peripheral blood at 30, 60 and 90 days post-transplant (dpt), and compared to data obtained in wild type and nontransplanted PKD littermates. A marked improvement in red blood cell counts was observed in PKD animals transplanted with genetically corrected cells HSCs as soon as 30 dpt. This recovery was stable for up to 90 dpt and was not observed in the animals transplanted with cells transduced with the control vector (**Figure 1a**).

High reticulocyte counts, a pathognomonic sign of PKD in patients, also occur in PKD mice. Through flow cytometry, we observed a steady reduction in reticulocytosis in PKD animals transplanted with genetically corrected cells, but not in PKD animals transplanted with cells transduced with the control vector (**Figure 1b**). This reduction was observed at 30 dpt and normal reticulocyte counts were recorded in subsequent analyses (**Figures 1b,c**).

To establish whether gene therapy was capable of restoring other red blood cell variables, hemoglobin, hematocrit, erythrocyte

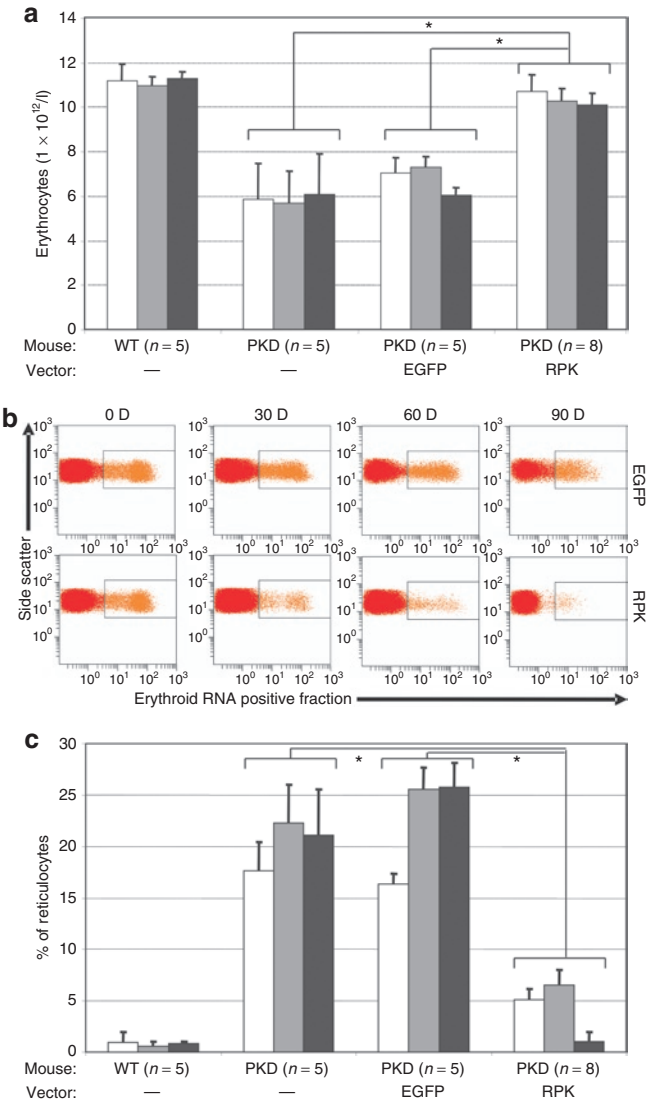

Figure 1 Erythrocyte and reticulocyte levels in animals transplanted with transduced cells. (**a**) Bars corresponds to peripheral blood erythrocyte levels in each group of pyruvate kinase deficiency (PKD) animals transplanted with enhanced green fluorescent protein or R-type pyruvate kinase at 30 (white bars), 60 (gray bars), and 90 (black bars) days post-transplant. Control groups of wild type and PKD littermates were analyzed in parallel. In brackets appears the number of animals analyzed per group. (**b**) Representative dot plot of the analysis used to monitor reticulocytes in peripheral blood. Upper row corresponds to a PKD deficient animal transplanted with cells transduced with the control vector. Lower row corresponds to a PKD mouse transplanted with cells transduced with the therapeutic vector. (**c**) Reticulocyte percentages recorded in the different groups of animals analyzed in **a**.

mean corpuscular volume and plasma iron levels were determined at 90 dpt. As shown in **Table 1**, these variables were restored in PKD mice transplanted with genetically corrected cells. In contrast, in animals transplanted with cells transduced with the control vector the abnormal red cell phenotype was not corrected.

Because of the hemolytic process, PKD patients require constant erythrocyte replenishment and this renders high serum levels of erythropoietin (EPO). We examined this factor in control and deficient animals, as well as in animals transplanted with transduced cells. Plasma EPO concentrations were up to 10 times

Table 1 Hematological variables recorded in retroviral transduced RPK-deficient mice

Animals[a]	Hg level (g/dl)	Hct	MCV (fl)	Plasma Fe level (μg/dl)	Plasma erythropoietin (pg/dl)
WT (n = 5)[b]	13.6 ± 0.75	47.8 ± 5.06	45 ± 2.00	251.8 ± 62.50	20.8 ± 10.22
PKD (n = 5)	8.7 ± 1.06	33.3 ± 2.51	54 ± 0.70	94.8 ± 6.04	163.7 ± 16.45
EGFP (n = 5)	9.1 ± 0.70	32.4 ± 1.89	52.5 ± 1.27	95.3 ± 9.29	144.7 ± 14.48
RPK (n = 8)	12.2 ± 0.51*	43.7 ± 2.43*	43.4 ± 1.51*	220.2 ± 65.70*	14.7 ± 14.17*

Abbreviations: EGFP, enhanced green fluorescent protein; PKD, pyruvate kinase deficiency; RPK, R-type pyruvate kinase; WT, wild type.
[a]WT, wild-type nonmanipulated mice; PKD, PKD nonmanipulated mice; EGFP, PKD mice transplanted with cells previously transduced with the EGFP expressing control vector; RPK, PKD mice transplanted with cells previously transduced with the human RPK expressing therapeutic vector. [b]Number of mice analyzed.
*statistically significant differences with respect to the PKD and EGFP groups, $P < 0.05$.

higher in both deficient animals and deficient animals transplanted with cells transduced with the control vector, compared to normal control littermates (Table 1). However, animals transplanted with RPK-corrected cells, had normal EPO concentration in plasma. Splenomegaly, another characteristic sign of PKD, was also examined in PKD mice at 110 dpt. Animals were killed and their spleens excised for morphometric analysis. As shown in Figure 2, PKD animals transplanted with genetically corrected HSCs showed a marked reduction in spleen weight and size. As expected, anemic mice transplanted with cells transduced with the non-therapeutic vector showed no reduction of splenomegalia.

The phenotypic changes observed were associated to the increase in activity levels of PK enzyme and ATP in erythroid cells (Table 2). In animals genetically corrected, the erythrocyte PK activity rose ~3.5-fold of the quantified in PKD mice (~75% of wild-type erythrocyte PK activity), and the ATP levels were restored to normal values. Collectively, these data indicate that transgenic expression of the human RPK protein completely resolves the hematological symptoms of this PKD mouse model.

Additionally, we evaluated iron accumulation in the liver by a Perls' reaction. Due to the hemolytic anemia that PKD animals suffer, there is an iron overload in liver and spleen. We observed that the correction of the disease by gene therapy was able to clear or reduce iron overload in the liver of animals transplanted with cells transduced with the therapeutic vector (Figure 2c). In contrast the animals transplanted with cells transduced with the control vector maintain high amounts of iron deposits in the liver.

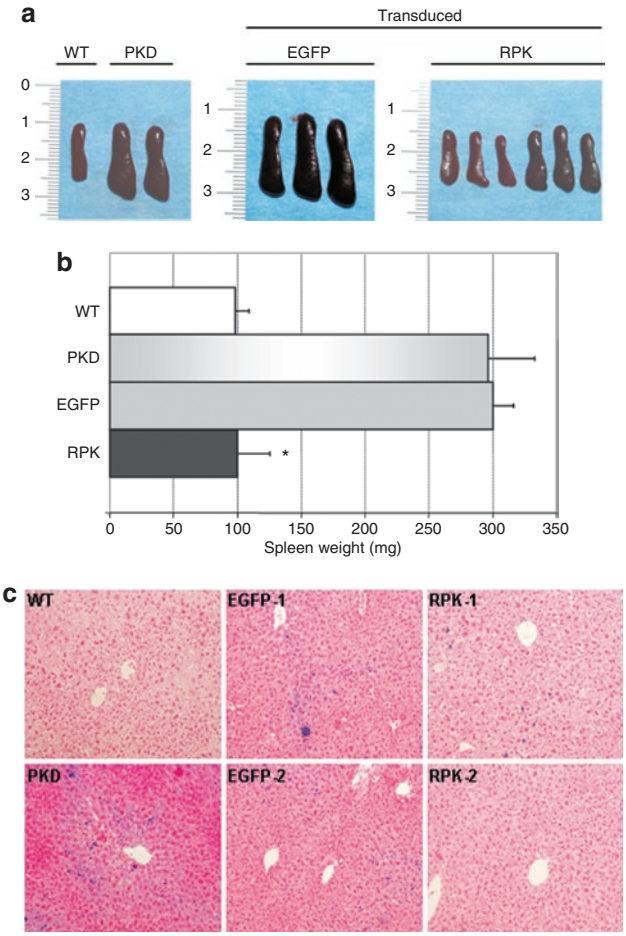

Figure 2 Morphometric analysis of spleens and iron deposits in the liver from primary recipients. (**a**) Pictures of representative spleens from each group of pyruvate kinase deficiency (PKD) animals transplanted with bone marrow cells transduced with enhanced green fluorescent protein (EGFP) or R-type pyruvate kinase (RPK) vectors. One spleen from a wild-type mouse and two spleens from PKD deficient mice (PKD) are also shown. (**b**) Average spleen weights for each group of PKD transplanted mice. (**c**) Photomicrographs of Perl's staining to identify iron deposits in liver sections from the same groups of animals shown in **a** and **b**. Sections from two different animals are shown for the groups EGFP (EGFP-1, EGFP-2) and RPK (RPK-1, RPK-2).

RPK expression in late erythroid precursors is needed to correct erythrocyte maturation and generate normal erythroid cells

Next, we examined differentiation patterns of the erythroid cell lineage in control and genetically corrected PKD animals. Expression of TER119 and CD71 antigens in spleen and BM cells were analyzed by flow cytometry. Four erythroid subpopulations: I, early proerythroblasts (Ter119^{med}CD71^{high}); II, basophilic erythroblasts (Ter119^{high}CD71^{high}); III, late basophilic and polychromatophilic erythroblasts (Ter119^{high}CD71^{med}); and IV, orthochromatophilic erythroblasts and mature erythroid cells (Ter119^{high}CD71^{low}) could be identified as previously described[23] (Figure 3a). A predominance of immature erythroid precursor cells (proerythroblasts, basophilic erythroblasts, and polychromatophilic erythroblasts) was identified in spleen and BM from PKD mice (Figure 3b). As expected, late erythroid populations were significantly low in PKD mice. The different erythroid subpopulations in BM and spleen were stabilized to normal ranges in animals transplanted with genetically corrected cells but not in those transplanted with cells transduced with the control vector (Figure 3b).

To identify at which erythropoiesis stage of the erythropoiesis anomalies occur in this PKD mouse model and whether altered

Table 2 Pyruvate kinase activity and ATP levels in genetically corrected RPK-deficient mice

Animals[a]	Erythroid PK activity (μkat/μg of protein)	Erythroid ATP level (nmol/μg of protein)	Leukocyte PK activity (μkat/μg of protein)	Leukocyte ATP level (nmol/μg of protein)	Serum PK activity (μkat/l)
WT ($n = 5$)[b]	$6.01 \times 10^{-4} \pm 7.0 \times 10^{-5}$	20.79 ± 2.42	0.028 ± 0.017	$16.1 \times 10^{-3} \pm 4.6 \times 10^{-3}$	0.47 ± 0.02
PKD ($n = 5$)	$1.29 \times 10^{-4} \pm 4.7 \times 10^{-5}$	13.89 ± 4.39	0.024 ± 0.004	$5.3 \times 10^{-3} \pm 2.4 \times 10^{-3}$	0.24 ± 0.09
EGFP ($n = 5$)	$1.16 \times 10^{-4} \pm 4.1 \times 10^{-5}$	15.77 ± 2.1	0.023 ± 0.007	$6.6 \times 10^{-3} \pm 3.1 \times 10^{-3}$	0.27 ± 0.12
RPK ($n = 8$)	$4.53 \times 10^{-4} \pm 1.7 \times 10^{-4}$*	29.36 ± 8.95*	0.034 ± 0.012	$13.23 \times 10^{-3} \pm 6.2 \times 10^{-3}$*	0.51 ± 0.21*

Abbreviations: ATP, adenosine triphosphate; EGFP, enhanced green fluorescent protein; PKD, pyruvate kinase deficiency; RPK, R-type pyruvate kinase; WT, wild type.
[a]WT, wild-type nonmanipulated mice; PKD, PKD nonmanipulated mice; EGFP, PKD mice transplanted with cells previously transduced with the EGFP expressing control vector; RPK, PKD mice transplanted with cells previously transduced with the human RPK expressing therapeutic vector. [b]Number of mice analyzed.
*statistically significant differences with respect to the PKD and EGFP groups, $P < 0.05$.

dynamics of erythroid precursor maturation could be rescued by RPK gene therapy, maturation dynamics of late erythroid precursors were studied in BM and spleen (**Figure 4**). Maturation dynamics were calculated as the variation in the percentage of precursors corresponding to two consecutive development stages (relative cell variation), as previously reported[23,27] and also detailed in Material and Methods. In the BM and spleen of PKD mice, relative increases of 22 and 3%, respectively, were observed in the shift from the basophilic to polycromatophilic erytrhroblasts and a similar relative cell loss between the polycromatophilic and the orthochromatophilic erythroblasts was detected. Overall, in this PKD model, the proliferative increase observed from basophilic (II) to polycromatophilic erytrhroblasts (III), mainly in BM, does not offset the cell loss from polycromatophilic (III) to orthochromatophilic erythroblasts (IV), suggesting that the expression of the erythrocyte PK isoenzyme is essential in the two last differentiation steps of the erythrocyte maturation (**Figure 4a**).

From the analysis of the enhanced green fluorescent protein (EGFP) expressing population in both groups of animals, we observed that the corrected cells were enriched in the progression of cells from step II to step III (**Figure 4b**), suggesting a differentiation advantage of corrected cells at this step of the erythropoietic differentiation. These findings support the impact of the therapeutic expression of RPK on the final differentiation and survival of the erythroid precursors.

Overexpression of the RPK protein does not affect metabolic variables in nonerythroid cells

Because of the use of a retroviral promoter, high levels of functional human RPK enzyme were expected not only in erythroid cells but also in other hematopoietic lineages. Analysis of EGFP expression in peripheral leucocytes at 30 dpt showed values around 50% and 40% of white blood cells from animals transplanted with cells transduced with SF11XEG or SF11RPKXEG vectors, respectively, expressing the transgene (data not shown). Increased levels of the PK protein could modify the energy production of blood cells expressing the transgenic human RPK. We thus determined PK activity and ATP levels in both erythrocytes and leukocytes in the different groups of animals (**Table 2**). As expected, erythrocytes from mice transplanted with genetically corrected cells showed normal PK activity and ATP levels. Interestingly, the different groups of animals showed no significant differences in leukocyte PK activity. Similarly, no differences emerged between leukocyte ATP levels in control mice and mice transplanted with corrected cells, indicating that the transgenic expression of human

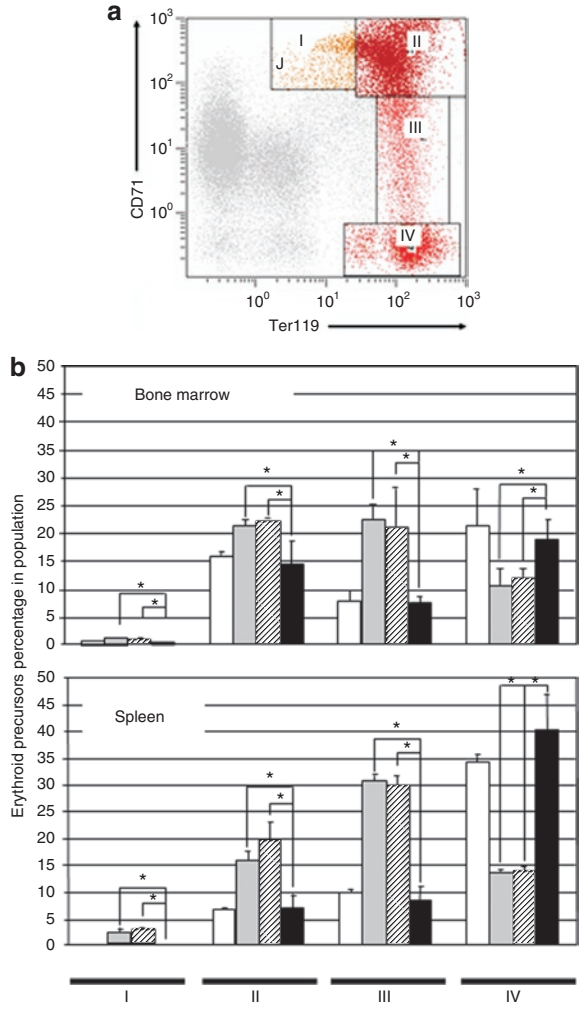

Figure 3 Flow cytometry analysis of erythroid precursor populations in the bone marrow and spleen of animals transplanted with transduced cells. (**a**) Representative dot plot and analysis of the different erythroid differentiation steps in bone marrow and spleen. Region I: proerythroblasts; Region II: basophilic erythroblasts; Region III: late basophilic and polychromatic erythroblasts; Region IV: orthochromatic erythroblasts and mature erythroid cells. (**b**) Percentage of each erythroid precursor population in pyruvate kinase deficiency (PKD) mice transplanted with enhanced green fluorescent protein or R-type pyruvate kinase transduced cells. The bone marrow and spleen of untransduced PKD mice and wild type mice were used as controls. White bars, wild-type mice; gray bars, PKD mice; hatched bars, deficient mice transplanted with cells transduced with the control vector; black bars, deficient mice transplanted with cells transduced with the therapeutic vector. *$P < 0.01$.

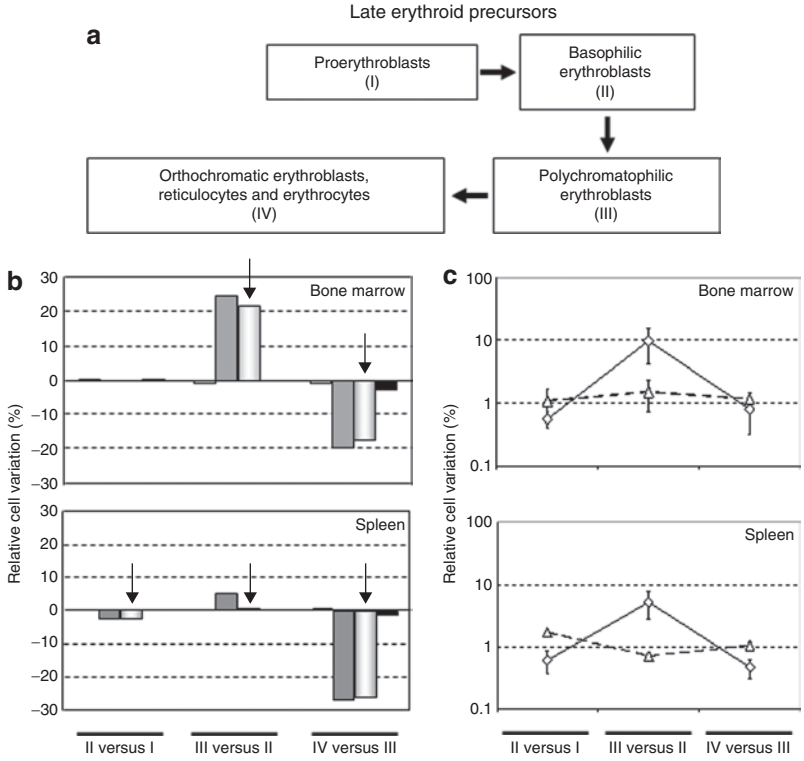

Figure 4 Dynamics of eythrodifferentiation in the bone marrow and spleen of animals transplanted with transduced cells. (**a**) Schematic diagram of erythroblast maturation indicating in roman numerals the populations studied in **b** and **c**. See also **Figure 3** for flow cytometry analysis of these populations (**b**) Relative cell variation between two consecutive erythroid precursor populations is indicated. Steps I–IV are referred to the populations established in **Figure 3**. Positive and negative values indicate an increase or decrease in cellularity between two consecutive differentiation steps. White bars, wild-type mice; gray bars, PKD mice; hatched bars, deficient mice transplanted with cells transduced with the control vector; black bars, deficient mice transplanted with cells transduced with the therapeutic vector. (**c**) Proliferative variation observed between two successive erythroid EGFP$^+$ populations in mice transplanted with cells transduced with the control vector (open triangle) or with the therapeutic vector (open diamond). EGFP, enhanced green fluorescent protein.

RPK in these cells did not affect the overall balance of the energy production pathway, probably due to fine regulation of this pathway by other key enzymes, intermediary metabolites and redox coenzymes. Moreover, numbers of peripheral blood leukocytes in genetically corrected mice were not affected, as compared with animals transplanted with the control vector (data not shown).

To check whether the transgenic expression of the human RPK could increase overall plasmatic enzyme activity, we also estimated PK levels in serum samples from the different animal groups. Again, normal levels of serum PK were observed, demonstrating the complete recovery of homeostasis and a lack of side effects in leukocytes produced by the increased PK expression (**Table 2**).

Long-term correction of the disease is dependent on the level of chimerism

Secondary transplants were performed to explore the stability and efficacy of phenotypic correction. Later 100 days primary transplantation, two-million pooled total BM cells from primary corrected recipients were transplanted into secondary RPK-deficient lethally irradiated mice (n = 7) and erythroid variables monitored in peripheral blood. A mean of 85% of genetically corrected myeloerythroid progenitors, as measure by provirus real-time quantitative PCR (qPCR) detection on methyl cellulose colonies, were transferred to each mouse. In these secondary recipients, erythrocyte and reticulocyte counts were normal up to 120 days postsecondary transplant (**Figure 5**, group RPK 100%) In addition, hematological variables remained normal, including PK activity in peripheral blood erythrocytes (**Supplementary Table S1**).

To determine the minimum number of corrected cells needed to achieve a therapeutic effect in our model, reduced numbers of corrected cells from primary recipients were transplanted into secondary recipients. Grafts of two-million cells containing 10% (~8.5% of genetically corrected cells as analyzed by qPCR) and 30% (~25% of genetically corrected cells) of cells from primary genetically corrected recipients, groups RPK 10 and 30%, respectively, were transplanted into secondary irradiated recipients (three mice per group). Shortly, after secondary transplant, groups RPK 10 and 30% displayed low numbers of peripheral erythrocytes but reduced numbers of reticulocytes (**Figure 5**). However, at 90 and 120 dpt this discrete reticulocytosis correction was lost (**Figure 5** and **Supplementary Table S1**). Taking into account that the higher number of corrected cells transplanted without a reverse in the anemic phenotype was 30% (25% corrected cells), we propose that values higher than those would be needed, at least, to rescue RPK-deficient animals from clinical symptoms.

Additionally, when pooled BM from fully corrected secondary animals was again transplanted into tertiary female PKD animals (n = 5), erythroid variables were fully restored up to 2 months

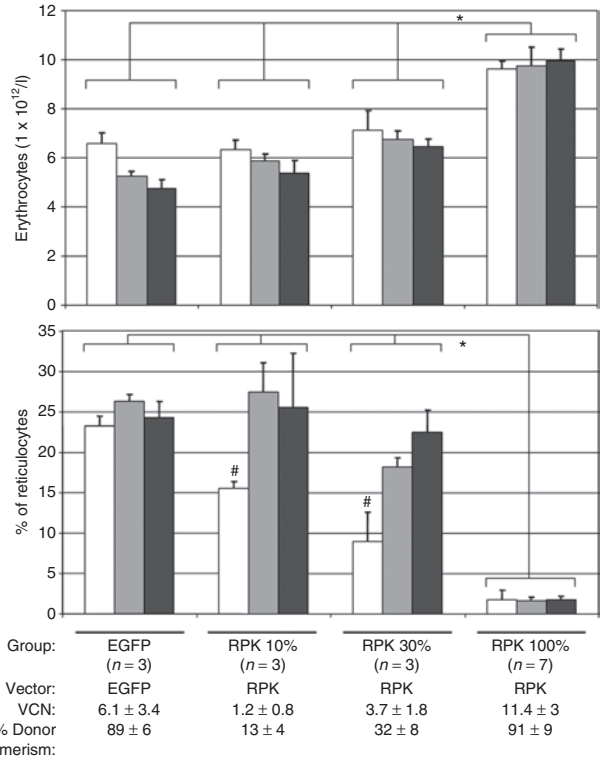

Figure 5 Analysis of erythrocyte and reticulocyte levels in secondary recipients. Bars correspond to (**a**) peripheral blood erythrocyte numbers, and (**b**) percentage reticulocytes for each group of pyruvate kinase deficiency recipients. Three groups with different percentages of primary derived hematopoietic cells (10, 30, and 100%) were evaluated at 30 (white bars), 60 (gray bars), and 90 (black bars) days post-transplant (dpt). A control group of wild type nonmanipulated littermates was also analyzed. In brackets, the number of animals studied per group is given. VCN, number of integrated viral DNA copies per cell analyzed by quantitative PCR. *Statistically significant differences between marked groups, $P < 0.05$. #Statistically significant referred to 60 and 90 dpt.

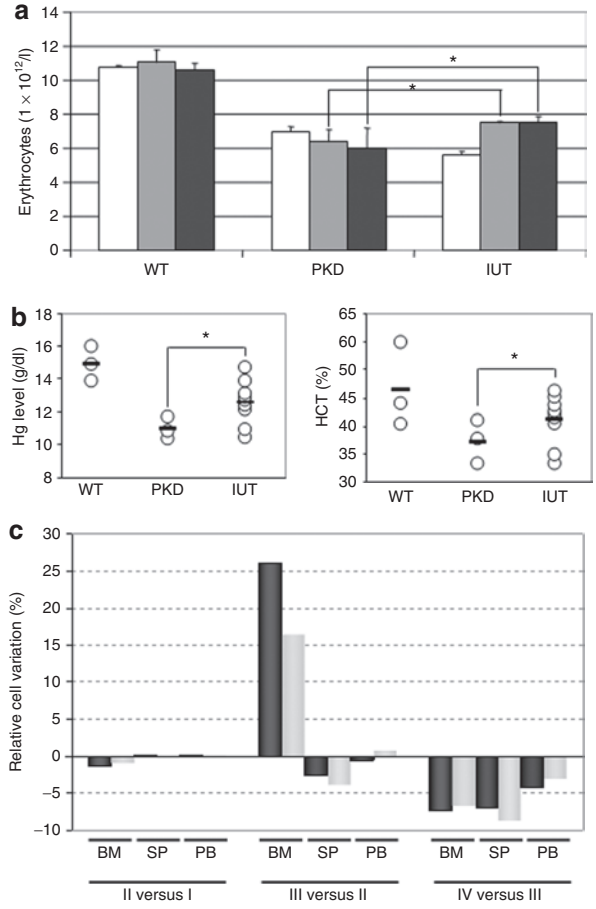

Figure 6 Partial correction of the erythropoietic phenotype in pyruvate kinase deficiency (PKD) mice transplanted *in utero* with genetically corrected cells. (**a**) Bars correspond to the number of peripheral blood erythrocytes detected in wild type, PKD and *in utero* transplanted mice at 30 (white bars), 60 (gray bars) and 90 (black bars) days postbirth (dpb). (**b**) Erythrocyte, hemoglobin, and hematocrit values recorded in PKD mice transplanted *in utero* with genetically corrected cells analyzed 90 dpb. WT, wild-type mice; PKD, PKD mice; IUT, mice *in utero* transplanted with genetically corrected bone marrow (BM) cells. (**c**) Percentage of cell variations between two consecutive erythroid precursor populations in BM (BM), spleen (SP) and peripheral blood (PB) from the same *in utero* transplanted animals. Black bars, PKD animals; gray bars, animals *in utero* transplanted with cells transduced with the therapeutic vector. *Statistically significant differences referred to PKD mice, $P < 0.05$.

after transplantation (data not shown). This data further demonstrates the transduction of very primitive HSCs and the stability of transgene expression in the deficient cells. Moreover, no evidence of leukaemia or myelodisplastic syndrome was observed in primary, secondary, and tertiary transplanted animals followed by flow cytometry and anatomopathologic studies (data not shown).

In utero gene therapy partially rescues anemia in RPK-deficient mice

We next tried to determine whether the treatment of this disease as early as possible might provide additional benefits over the improved clinical manifestations of PKD noted in the treated adult animals. To this end 14.5 day-old fetuses from pregnant PKD female mice were transplanted with 5×10^5 genetically corrected hematopoietic progenitors/fetus. Erythroid variables were followed up to 90 days after birth (Figure 6). At 30 days postbirth, erythrocyte counts were low. However, increased numbers were recorded at 60 days postbirth and these numbers were steady at 90 days postbirth (Figure 6a). Consistently, hematocrit and hemoglobin levels showed similar behavior indicating partial correction of anemia in *in utero* transplanted animals (Figure 6b). Analysis of late erythroid precursor dynamics showed a partial correction in the BM in the *in utero* transplanted animals in comparison with deficients animals of the same age (Figure 6c).

To estimate the engraftment efficiency of corrected cells, we determined the number of proviral insertions in BM by quantitative real-time PCR. A mean of 0.8% of transduced $EGFP^+$ cells was detected in the *in utero* transplanted animals 3 months after birth. It is worthy to point out that similar partial corrections were observed in the *in utero* transplanted animals than in the secondary recipients transplanted with tenfold more corrected primary cells (group RPK 10%).

DISCUSSION

The treatment of genetic erythroid deficiencies, such us hemoglobinopathies, or metabolic diseases like glucose-6-phosphate dehydrogenase deficiency by gene therapy has yielded promising

results in animals models.[28] Here, we demonstrate that our previously developed retroviral vectors[23] are capable of fully resolving the pathological symptoms of PKD in a mouse model, including anemia, reticulocytosis, and splenomegalia, and of normalizing EPO, PK, and ATP levels, without modifying the metabolic energy balance in white blood cells. Moreover, we show that values above 25% of genetically corrected cells are needed to fully rescue the deficiency. The lack of sufficient therapeutic effects observed in adult chimerism and in *in utero* assays discarded a possible proliferative advantage from corrected HSC population. Also, we suggest that the *in utero* transplant of RPK-corrected cells could be a therapeutic strategy when fetal anemia due to PKD is early diagnosed to prevent *hydrops fetalis* due to this disorder.[29]

Transplantation of RPK-corrected cells resolved erythroid pathological symptoms as early as 30 dpt, indicating that the normal function of human RPK in corrected erythrocytes is rapidly restored. Splenomegalia and iron deposits in the liver, common signs of hemolytic anemia were also reversed in response to the gene therapy protocol, further demonstrating that the damage on these organs is also reversed after anemia rescue. As expected, other relevant erythroid variables, including hemoglobin levels, hematocrit, mean corpuscular volume and iron plasma levels, were fully restored in PKD animals transplanted with the therapeutic vector, demonstrating that the expression of human RPK in the mouse erythrocytes completely rescues this metabolic pathway in the erythroid lineage and allows the generation of normal erythrocytes in peripheral blood. In addition, EPO levels dramatically drop indicating that a normal steady state of hematopoiesis is attained after gene therapy.

Although the use of retroviral vectors to drive the ubiquitous expression of the *hRPK* gene could disturb the physiological generation of ATP in white blood cells, ATP levels measured in peripheral blood leukocytes from genetically corrected PKD mice were not increased as a result of the ectopic expression of the human RPK in these cells, indicating a fine regulation of this pathway either by direct ATP inhibition[30] or by metabolic and transcriptional control.[31]

In humans, pathological signs of PKD appear when enzyme activity drops below 25% normal levels.[32] A mouse model of PKD has revealed that 10% normal BM renders red blood cells expressing nearly normal RPK protein levels.[18] As a result of the transduction protocol used here, the viral dosage was high, up to 11 viral DNA copies per cell in the case of the group RPK 100%. However, due to the variability of the transplanted animals some mice with phenotypic correction receive no more than 2.5 viral copies per cell (data not shown). To estimate the minimal amount of genetically corrected cells needed for rescue therapy, we transplanted 30 and 10% of BM cells harvested from primary recipient animals, with 25 and 8.5% of corrected cells, respectively. Although in the short-term reticulocytosis was significantly corrected, these reduced proportions of transduced HSCs were unable to solve the hematological defects of PKD mice in the long term. These data indicate that, in our PKD mice model the relevant parameter to obtain a phenotypic correction is the amount of corrected cells transplanted.

Interestingly, a low correction of the phenotype was observed in PKD mice injected *in utero* with a 0.8% of engraftment of transduced cells. These data suggest that the administration of corrected cells at very early development stages could be therapeutically a more efficient strategy to address the correction of this disease. This strategy should be very efficient for PKD cases in which fetal symptoms are detected. In these cases fetal liver biopsys for the isolation of hematopoietic progenitors[33,34] that could be genetically corrected and reinfused into the fetus could be applied.

In gene therapy for metabolic diseases, the expression of the normal version of the dysfunctional protein could confer the corrected cells an advantage over noncorrected cells. In our system, we observed an increased capacity of basophilic erythroblasts to differentiate into polychromatic erythroblasts, suggesting the dependence of this step on correct RPK expression. Thus, the expression of the human RPK enzyme in deficient erythroid precursors confers a significant differentiation advantage over noncorrected deficient erythroid precursors. However, when reduced numbers of corrected HSC are transplanted as occurred in secondary transplants of the group RPK 10%, or when corrected cells were transplanted *in utero*, the therapeutic effect is lost. These evidences would indicate that the differentiation advantage observed in the genetically corrected erythroid precursors might be insufficient to completely rescue the deficiency in the presence of low numbers of corrected cells. Alternatively, the transfer of reduced numbers of cells could result in the absence of genetically corrected hematopoietic stem cells in the graft that will end in the loss of anemia recovery in the long term.

Attempts to selectively expand normal donor erythrocytes have included the use of chemical inducers of dimerization in minimally conditioned, BM transplanted pyruvate kinase-deficient mice, but the proliferative advantage observed in normal erythroid progenitors was found to be dependent on regular chemical inducers of dimerization administration.[18] Thus, RPK transfer protocols will always require a significant extent of donor chimeric hematopoiesis. In our system, high and stable expression levels of the *RPK* gene after myeloablative conditioning *in vivo* was achieved, with no deleterious effects observed even after tertiary transplant. The use of autologous genetically corrected cells along with a submyeloablative conditioning protocol to allow significant engraftment of genetically corrected HSCs, as it is undertaken in adenosine deaminase deficient patients,[35] would probably be sufficient for successful *RPK* gene therapy in humans.

Insertional mutagenesis leading to leukaemia has been associated with the transactivation of oncogenes due to insertion of retroviral vectors in the vicinity of the affected genes.[36,37] Although we have not observed adverse effects associated to the gene therapy protocol up to tertiary transplanted animals, improved vectors such as self-inactivated retroviral vectors, either γ- or lenti-vectors, which have shown lower risks of leukaemogenesis,[38,39] should be used. In addition, the obvious benefits of lineage-specific transgene expression determine that the ideal vector for RPK gene therapy would be a self-inactivating lentiviral vector carrying an erythroid-specific promoter[40] or, at least, a weak promoter driving the expression of the *hRPK* gene. We have recently evaluated the potential use of weak promoters such as the *vav* proto-oncogene, which shows weak and stable protein expression.[41] New lentiviral vectors using this promoter for the treatment of PKD are presently being developed in our laboratory.

In summary, we show here that murine erythroid PKD can be treated by gene therapy with HSCs. Due to its long lasting nature and the fact that it solves all hematological symptoms, this strategy may be an option for the management of severe cases of PKD patients (RPK activity below 20% of normal levels) in which splenectomy and periodical red cell infusions fail to control the anemia and the only therapeutic alternative should be allogeneic BM transplant. Patients with these characteristics lacking a human leukocyte antigen matched donor should be the ideal target. Also, *in utero* transplantation of corrected cells could be a helpful approach in order to diminish the severe fetal and childhood clinical manifestation, such as *hydrops fetalis,* stillbirth and early neonatal death once a clinically relevant *in utero* engraftment could be obtained.

MATERIALS AND METHODS

Animals. AcB55 (C57Bl/6 × A/J) mice (also recorded as PKD mice) carrying a point mutation at nucleotide 269 (T→A) of the *pklr* gene[16] were obtained from Emerillon Therapeutics (Montreal, Quebec, Canada) and bred at the animal house of the CIEMAT (registration number 28079-21A). Animals were allowed food and water ad libitum, and were routinely screened for pathogens in accordance with Federation of European Laboratory Animal Science Associations procedures. All experimental procedures were conducted according to Spanish and European legislation (Spanish RD 223/88 and OM 13-10-89 of the Ministry of Agriculture, Food and Fisheries, for the protection and use of animals in scientific research; and European convention ETS-123, for the use and protection of vertebrate mammals used for experiments or other scientific purposes). Eight-to-ten week-old PKD mice were used as BM cell donors and recipients.

Vectors and cell lines. The SF11XEG vector, expressing the EGFP, and SF11RPKXEG vector, expressing the human RPK and the EGFP proteins in a single mRNA transcript containing an internal ribosome entry site sequence between both cDNAs, vectors used here are modifications of pSF11γ (GenBank accession no. AJ132035) as described previously.[23] These vectors contain the 3′ LTR of spleen focus-forming virus and the leader sequence of murine embryonic stem cell virus.[42] Phoenix 293T cell-based ecotropic packaging cells (Phoenix-eco; kindly provided by Dr Nolan, Stanford University, Stanford, CA) were maintained in Dulbecco's modified Eagle's medium (Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum (Intergen, Purchase, NY).

Viral supernatant production and cell line transduction. To establish ecotropic packaging cells for the SF11XEG and SF11RPKXEG vectors, 20 µg of the corresponding retroviral plasmid DNA were transfected using FuGENE reagent (Roche Diagnosis, Indianapolis, IN) on Phoenix-eco cells following the manufacturer's recommendations. $EGFP^+$ cells were sorted in an EPICS Elite ESP flow cytometer (Coulter Electronics, Hialeah, FL) and kept as producer pools.

Purification, transduction, and transplant of murine HSCs. BM was harvested from 8 to 10 weeks old PKD male mice and subjected to red blood cell lysis. Lin^- progenitors were sorted using the Lin^- magnetic sort kit (Miltenyi Biotec, Gladbach, Germany) according to the manufacturer's instructions. On average, 80–90% pure populations of Lin^- were obtained. Transduction of fresh Lin^- cells were performed as previously described.[23] Briefly, Lin^- cells were cultured *in vitro* for 48 hours in Iscove's Modified Dulbecco's Medium (BioWhitacker, Walkersville, MD) plus 20% fetal bovine serum, 100 ng/ml murine stem cell factor and 100 ng/ml human interleukin-11. Cells were then harvested and resuspended in fresh supernatant from retroviral producer cells supplemented with the same growth factors and plated in retronectin (Takara, Shiga, Japan) coated dishes previously loaded with the corresponding retroviral vectors (3 incubations for 30 minutes of freshly supernatants). Hematopoietic cells were cultured for another 48 hours with 12 hour changes of viral supernatant containing factors. Cells harvested 4 hours after the last infection cycle were washed twice in phosphate-buffered saline (1× + 0.1% bovine serum albumin + 0.02% NaN_3), and 2×10^5 hematopoietic cells/mouse were injected into the tail vein of 8 to 12 week-old female PKD mice, previously irradiated with 9.5 Gy in two split doses of 4.75 Gy, 24 hour apart, using a Philips MG324 X-ray instrument (Philips, Hamburg, Germany) set at 300 kV, 10 mA, and a delivery dose rate of 1.03 Gy/minute.

Hematological variables. Anticoagulated blood samples were collected for haematology analysis and to determine PK activity, ATP, and EPO. Complete blood counts, hematocrits, hemoglobin contents, and red blood cells indices were obtained using an automated blood cell analyzer (Abacus Junior vet; CMV Analitica, Spain). PK activity was determined by the lactate dehydrogenase-coupled spectrophotometric assay[43] using the PKD Kit (Greiner diagnostic, Bahlingen, Germany). PK activity was expressed as microkatals per microgram of total protein (µKat/µg protein). Basal rates of activity were measured at all times.

ATP was quantified using a spectrophotometric ATP detection assay system (ATP Hexokinase FS; DiaSys, Holzhelm, Germany). This assay is based on the reaction of glucose with ATP catalyzed by hexokinase and Mg^{2+} ions. ATP concentration was expressed as micromoles per microgram of total protein (µmol/µg protein). Plasma EPO concentrations were determined through an enzyme-linked immunosorbent assay to detect mouse EPO using an anti-mouse EPO antibody (Quantikine Mouse Epo Immunoassay; R&D Systems, Minneapolis, MN).

Colony-forming unit cell assays. BM-derived cells (3×10^4) were seeded on 35-mm plastic tissue culture dishes (Nunc, Roskilde, Denmark) in triplicate in methyl cellulose (MethoCult GF M3534 culture medium, StemCell Technologies, Vancouver, British Columbia, Canada) containing recombinant murine stem cell factor (50 ng/ml), recombinant murine interleukin-3 (10 ng/ml), recombinant murine interleukin-6 (20 mg/ml), recombinant murine granulocyte-macrophage colony-stimulating factor (granulocyte macrophage–colony stimulating factor; 0.01 ng/ml), and EPO (recombinant mouse EPO; 3 U/ml). Cultures were incubated at 37 °C in a 95% humidified atmosphere with 5% CO_2 in air. Later 7 days plating, overall colony numbers and $EGFP^+$ colonies were scored under a light and epifluorescence microscope, respectively.

Flow cytometry and quantification of cell variation at specific erythroid subpopulation. The transgene expression and variation in the percentage of erythroid precursors in each erythroid development stages was measured as described elsewhere.[23,27] Briefly, to analyze the most related erythroid precursors obtained from BM and spleen, 1×10^6 cells were stained with biotinylated anti-CD71 murine (Pharmingen, Palo Alto, CA) and TER-119-phycoerythrin (PE) (Pharmingen) antibodies, washed and incubated with streptavidin-tricolor (Caltag, Burlingame, CA). Cells were resuspended in phosphate-buffered saline with 2 µg/ml propidium iodide, and analyzed. A minimum number of 3×10^5 viable cells were acquired using a EPICS XL flow cytometer (Coulter Electronics, Hialeah, FL).

Quantification of cell variation at specific subpopulation stages was calculated following the mathematical development described elsewhere.[27] Percentage of cell gain or loss (CL) in a subpopulation was calculated using the equation:

$$\mathrm{CL} = 1 - \frac{P^{\exp_{i+1}} \times P^{\mathrm{control}_i}}{P^{\exp_i} \times P^{\mathrm{control}_{i+1}}} \times 100$$

Where P^{control_i} is the percentage of cells in the initial population in control mice, $P^{\exp_i}$ the percentage of cells in the initial population in RPK-deficient mice and $P^{\mathrm{control}_{i+1}}$ the percentage of cells in the subsequent population in

controls. To calculate the relative cell variation between two stages the following formula was applied: relative cell variation = $-(CL \times P^{exp}{}_i/100)$, where a positive value corresponded to relative cell gain and a negative value corresponded to a relative cell loss. For reticulocyte analysis, 5 µl total blood was added to 2 ml phosphate-buffered saline containing 0.1 µg/ml of Acridine Orange (Invitrogen, Carlsbad, CA), incubated for 30 minutes at room temperature and directly analyzed by flow cytometry. Reticulocytes were identified as RNA positive cells within the erythrocyte population. A total of 10^6 erythrocytes were analyzed.

Chimerism. Chimerism was quantified as described[44] through a real-time qPCR approach using Rotor Gene RG-3000 (Corbett Research Products, Foxboro, MA). Genomic DNA was extracted using the DNAeasy Tissue Kit (Qiagen, Duesseldorf, Germany) according to the manufacturer's instructions. Primers for the male-specific sequence were as follows: SRY-F: 5′-TGTTCAGCCCTACAGCCACA-3′ and SRY-R: 5′- CCTCTCACCACG GGACCAC and detected with the TaqMan probe SRY-T: 5′-FAM-ACA ATTGTCTAGAGAGCATGGAGGGCCA-BHQ1. The murine genomic β-actin sequence was amplified using the primers: β -actin-MF: 5′-AC GGCCAGGTCATCACTATTG-3′ and β -actin-MR: 5′-ACTATGGCCTC AAGGAGTTTTGTCA- 3′; and detected with the TaqMan probe β-actin-T: 5′-TR-AACGAGCGGTTCCGATGCCCT-BHQ2-3′. Real-time qPCR was carried out in a multiplex reaction. For Amplification, 5 µl of genomic DNA (to 5 ng/µl) were mixed with 20 µl of a PCR master mix consisting of 1 × TaqMan universal master mix NoAmpErase (UNG Applied Biosystems, Roche, Nutley, NJ), 200 nmol/l of each primer and 200 nmol/l of each probe. The thermal profile was one hold of 10 minute at 95 °C, and 55 cycles of 20 seconds at 95 °C, and 30 seconds at 58 °C.

Proviral DNA analysis. To monitor provirus copy numbers, the EGFP sequence from the retroviral engineered vectors was assayed in transduced cells by real-time PCR. Primers and the TaqMan MGB probe were designed with the aid of the Primers Express software program (Applied Biosystems, Carlsbad, CA). The specific EGFP detector was composed of the forward primer: F1EGFP 5′ GTAAACGGCCACAAGTTCAGC; reverse primer R1EGFP 5′ TGGTGCAGATGAACTTCAGGG and the TaqMan MGB probe PEGFP 5′ 6-FAM-CTTGCCGTAGGTGGC-MGB. For cell lines, DNA was isolated from 1×10^6 cultured cells or from 5×10^5 BM cells using the Puregene kit (Promega, Madison, WI). Genomic DNA was resuspended in 200 µl of TE Buffer (10 mmol/l Tris with 0.1 mmol/l EDTA), incubated at 65 °C for 30 minutes, vortexed, and stored at 4 °C. For real-time PCR analysis, 5 µl of genomic DNA (to 5 ng/µl) were mixed with 20 µl of a PCR master mix consisting of 1 × TaqMan universal master mix, 200 nmol/l of each primer (F1EGFP/R1EGFP) and 200 nmol/l of the MGB probe (PEGFP). For negative controls, we used 5 µl of H_2O and 5 µl of genomic DNA from untransduced cells. All reactions were conducted in triplicate and amplifications were performed as one cycle of 95 °C for 10 minutes, and 40 cycles of 95 °C for 30 seconds and 58 °C for 30 seconds. The average number of provirus copies per cell was quantified using a standard curve for the retroviral plasmid as described previously.[45]

To measure the transduction efficiency in hematopoietic progenitors we followed the protocol for Villella *et al.*[46] with several modifications. Single colonies growing in methylcellulose were harvested and transferred directly into the PCR tubes, containing 50 µl crude cell lysing buffer (1× PCR buffer, 0.5% NP-40; 0.5% Tween 20; and 0.91 mg/ml proteinase K). Approximately 80 colonies from each sample were collected and labeled. Nontranduced BM and methylcellulose samples obtained from noncolony areas of the plates and samples containing cell lysis buffer alone were analyzed as negative controls. After all colonies were transferred, the tubes were placed in a thermal for 1 hour at 60 °C followed by 15 minute at 95 °C. Then, to detect EGFP sequences from the retroviral engineered vectors, 10 microliters of cell lysate from each sample was then transferred to each corresponding tube previously loaded with 40 µl per well PCR master mix, consisting of 1 × TaqMan universal master mix, 200 nmol/l of each primer (F1EGFP/R1EGFP) and 200 nmol/l of the MGB probe (PEGFP). All reactions were conducted in duplicate and amplifications were performed as one cycle of 95 °C for 10 minutes, and 55 cycles of 95 °C for 30 seconds and 58 °C for 30 seconds. Real-time qPCR assay on the murine genomic β-actin sequence using similar thermal profile and primers/probe concentration was done as an internal control for DNA content. Samples that did not contain the β-actin sequence were removed from the analysis.

In utero transplantation. Retrovirally transduced Lin^- progenitors from PKD mice BM were injected in PKD fetuses on gestation day 14.5. Briefly, under isoflurane anesthesia, the uterine horns were exposed through a midline laparotomy. Under a dissecting microscope, the fetal liver was identified, and each foetus was injected with 5×10^5 Lin^- cells transduced with the human RPK oncoretroviral therapeutic vector in a total volume of 5 µl using a 100 µm bevelled-glass micropipette. After this, the uterine horns were returned to the maternal peritoneal cavity, the abdomen closed by two running reabsorbable 5-0 vicryl sutures and each mother injected subcutaneously with 0.15 mg/weight mg of buprenorphine (Buprex; Reckitt Benckiser Healthcare, Hull, UK) as analgesic. After birth, hematological variables were monitored for up to 3 months in the surviving offspring.

Statistical analysis. Data are represented as the mean ± standard deviation of the mean. The significance of differences between groups was determined by using the nonparametric Wilcoxon Mann–Whitney *W* test. The processing and statistical analysis of the data was performed by using the Statgraphics Plus 5.0 software package (Manugistics, Rockville, MD).

SUPPLEMENTARY MATERIAL

Table S1. Relevant hematological variables recorded in secondary PKD recipients of genetically corrected BM cells obtained from transduced RPK-deficient mice.

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Development of an *in vitro* model for the simultaneous study of the efficacy and hematotoxicity of antileukemic compounds

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ABSTRACT

Hematopoietic system displays a wide spectrum of cell populations hierarchically organized in the bone marrow. Homeostasis in this system requires equilibrium between the self-renewal of the stem cells and their capacity of differentiation. Any failure on this equilibrium could lead to fatal consequences, such as the development of leukemia. Due to its rapid rate of renewal, hematopoietic tissue is a major target for antitumoral compounds and often becomes a dose limiting factor in the development of antineoplastics. Our aim was to develop an *in vitro* model for predicting the efficacy of antitumoral compounds on leukemic cells and their toxic effects on the healthy hematopoietic cells.

The mouse myelomonocytic leukemia WEHI-3b was transduced with a lentiviral vector for expressing the green fluorescence protein. Mixed semisolid clonogenic cultures of transduced WEHI-3b and murine bone marrow cells were exposed to five pharmaceuticals: daunorubicin (positive control), atropine sulphate (negative control) and three in different stages of clinical development (trabectedin, Zalypsis® and PM01183). Colonies of leukemic cells were distinguishable from healthy CFU-GM under fluorescence microscope. The sensitivity of leukemic cells to daunorubicin, trabectedin, Zalypsis® and PM01183 was higher compared to healthy cells. The effect of a non-antitumoral compound, atropine sulphate, was the same on both populations. Our results show that this *in vitro* model is a valuable tool for studying the effect of antitumoral compounds in both tumoral and normal hematopoietic cells under the same toxic microenvironment and could safe time and facilitate the reduction of the number of animals used in preclinical development of pharmaceuticals.

1. Introduction

Hematopoietic system displays a wide spectrum of cell populations whose constant proliferation and differentiation in the bone marrow (BM) give rise to erythroid, granulocytic, macrophagic, megakaryocytic and lymphoid blood cells. BM is an extremely complex tissue which is regulated by an equally complex set of hematopoietic growth factors and by specific interactions with the stromal cells of the hematopoietic environment. Hematopoietic tissue is hierarchically organized, a small number of stem cells gives rise to a whole variety of morphologically and functionally differentiated cells. The stem cell population is the fundamental base from which all the mayor blood lines are derived. The main property of this population is its ability to maintain its own number. Committed progenitors are derived from the stem cells and are committed to a particular line of cell development. They are transit cells that amplify the population prior to its production of functional maturation stages (Sachs, 1987; Lord and Testa, 1988; Morrison et al., 1995). Homeostasis in the hematopoietic system requires equilibrium between the self-renewal of the stem cells and their capacity of differentiation. Any failure on this equilibrium could lead to fatal consequences. One of these consequences is the development of leukemias (Zeleznik-Le et al., 1995; Bonnet and Dick, 1997).

The objectives of toxicology studies are the identification of potentially dangerous toxicants, so that human exposure can be prevented or controlled, and the provision of relevant information for undertaking risk-benefit analyses and for conducting clinical trials. Toxicants can cause hematotoxicity by interfering with mature blood cell, committed progenitors or stem cells functions or survival. Following cytotoxic insult, for instance after chemotherapy, neutropenia and trombocytopenia are often non-desirable clinical effects which make the hematopoietic system to be the limiting factor. For the preclinical development of antineoplastics, *in vivo* toxicology studies are generally conducted in at least two different species, employing various dosing schedules. These *in vivo* data are used to derive the dose for the first human exposure, which is

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usually at least 10 times lower than the maximum tolerated dose (MTD) in animals (DeGeorge et al., 1998; Sistare and DeGeorge, 2007).

Any *in vitro* test which can refine these safety margins, by reducing the toxicological uncertainties underlying laboratory animal/human extrapolations, would be of great benefit, since it would provide a more scientific and rational basis for calculating clinical dosages and for setting human exposure limits.

During the first stages of the preclinical development of an antitumoral, in order to get the first hints of effect on the hematopoietic tissue, studies on established hematopoietic cell lines are generally performed (HL-60, K562, etc.). Later on, *in vivo* hematological parameters are determined in different animal models. On the other hand, the effect of the compounds on tumoral cells is also studied, first with cell lines and then in animal models bearing artificial tumors. There are not steps in the process in which tumoral and normal cells are studied in the same model and under the same microenvironmental parameters. This is especially important in the case of leukemic cells which share a lot of characteristics with the healthy hematopoietic cells (Rosmarin et al., 2005; Misaghian et al., 2009).

In the present study, a new *in vitro* model is described. We have developed an *in vitro* assay in which, leukemic and normal hematopoietic progenitor cells, can growth simultaneously and be distinguished from each other. Focusing on a model for acute myeloid leukemia (AML), we have used WEHI-3b cells, as the leukemic population, and Colony Forming Units of Granulocytes and Macrophages (CFU-GM) progenitors from healthy murine BM. By means of this assay, the efficacy of antileukemic compounds on leukemic cells and, their toxic effect on normal hematopoietic progenitors, can be study in the same *in vitro* system, thereby allowing the growth under the same cytotoxic pressure. In order to facilitate the identification of leukemic colonies, WEHI-3b cells have been transduced with a lentiviral vector (LV) for expressing the green fluorescence protein. Colonies of transduced WEHI-3b cells were distinguishable from healthy CFU-GM colonies of murine BM in semisolid cultures under fluorescence microscopy. The use of this assay in early stages of the preclinical development could avoid surprises in later stages when a lot of animals, money and time could be misspent.

2. Materials and methods

2.1. Mice

Twelve to 14 week-old Balb/C mice were used as donors of bone marrow cells (BMC). Breeding pairs, originally obtained from the Jackson Laboratory (Bar Harbor, ME) were bred at the Centro de Investigaciones Energéticas, Medioambientales y Tecnológicas (CIEMAT) Animal Facility (Madrid, Spain; Registration No. 28079-21 A) and allowed food and water *ad libitum*. After euthanasia by carbon monoxide, femurs were aseptically removed, and the marrow was flushed from the central canal with Iscove's modified Dulbecco's medium (IMDM, GIBCO, Grand Island, NY) via syringe.

Recipients of BM grafts received total body irradiation with a fractionated dose of 8.34 Gy (2 doses of 4.17 Gy spaced 4 h apart; dose rate 1.03 Gy/min) using Philips MG 324 X-ray equipment (Philips, Hamburg, Germany), at 300 kV, 10 mA.

All the experiments performed with mice were conducted in accordance with the *Guiding Principles in the Use of Animals in Toxicology*, adopted by the Society of Toxicology in 1989 and the Guidelines for the welfare of animal in experimental neoplasia adopted by the UKCCCR (United Kingdom Coordinating Committee for Cancer Research).

2.2. Culture of WEHI-3b cells

WEHI-3b cells were obtained from ATCC (Manassas, VA, USA) and cultured in Dulbecco's Minimum Essential Medium (DMEM, Invitrogen, CA) supplemented with 10% Foetal Bovine Serum (FBS, Lonza, Belgium), 2 mM L-glutamine (Gibco BRL Life Technologies, Grand Island, NY), 100 U/mL penicillin, and 100 μg/mL streptomycin (Pen-Strep, Gibco) and kept at 37 °C in a humidified atmosphere with 5% CO_2. Periodically, cultures were tested for the presence of mycoplasm.

2.3. Clonogenic assays

Following the instructions of the manufacturer, an appropriate number of viable cells (murine BMC and WEHI-3b cells) were seeded in dishes containing MethoCult GF M3534 culture media (StemCell Technologies, Vancouver, BC) in the presence of different concentrations of drug and plated in triplicate on 35-mm plastic tissue culture dishes (Nunc, Denmark). The colonies were scored after 7 days at 37 °C in 5% CO2 and fully humidified air; the CFU-GM colonies were scored under an inverted microscopy following standard criteria (Pessina et al., 2001). At least, three independent experiments with a dose-survival response were performed for each compound.

2.4. Transduction of WEHI-3b cells

Lentiviral supernatants were obtained following the information provided as Supplementary data (Bacchetti and Graham, 1977; Graham and van der Eb, 1973; Laurent and Jaffrézou, 2001; Schöffski et al., 2007) For the transduction, WEHI-3b cells were resuspended in RPMI 1640 supplemented as previously described at a density of 10^5 cells/mL. Transduction was done in two 90 min spinoculation cycles spacing 24 h. Viruses were added at a multiplicity of infection (MOI) of 10. After the infection, cells were washed and resuspended in supplemented medium for culture.

2.5. Drugs

Daunorubicin was used as a positive control because it is a referent antitumoral compound for the treatment of hematological malignancies (Laurent and Jaffrézou, 2001). Atropine sulphate monohydrate was included as a negative control because it is a non-antitumoral compound. It is used in ophthalmology, before eye examinations to open the pupil, as well as to relieve pain caused by swelling and inflammation of the eye. Both compounds were purchased to Sigma–Aldrich (St Louis, MO, USA). Three new antitumoral compounds were also included, trabectedin (ET-743; *Yondelis*®), Zalypsis® and PM01183. Trabectedin is a potent antitumor drug currently undergoing phase II/III clinical trials that has recently received regulatory approval from the European Medicines Agency (EMEA) for the treatment of metastatic or advanced soft tissue sarcoma after failure to anthracyclines and ifosfamide (Schöffski et al., 2007). Zalypsis® is currently under late phase I development in solid tumors, with preliminary evidence of antitumoral activity (Ocio et al., 2009). Phase I clinical trials have been recently initiated with PM01183 (June 2009) for patients with advanced solid tumors. All these antitumoral compounds were provided by PharmaMar (Colmenar Viejo, Spain) as a dry powder to be reconstituted with dimethyl sulfoxide (DMSO, Sigma–Aldrich) and diluted to a concentrated stock solution (1 mM) and kept in aliquots at −80 °C. It was prepared fresh before each experiment.

Whenever possible, data of plasma levels and/or *in vitro* studies from literature were used as indicative doses of exposures *in vitro*. When these data were not available, a half-log drop schema was used to find the range with effect. In a second set, doses were chosen within the inhibitory concentration of 30% and 90% of growth (IC_{30}–IC_{90} range) in order to fine-tune the dose–response curve between critical concentrations.

2.6. Statistical methods

All the results were calculated as survival percentages respect to control cultures exposed to DMSO 0.1% (final concentration of drug vehicle in cultures). The survival data were fitted by least squares only for experiments with at least three available data points. The IC_{50}, IC_{70} and IC_{90} was obtained algebraically, solving the fitted quadratic equation for the value of dose where the estimated percentage of surviving cells would equal 50%, 30% and 10%, respectively. The estimated IC_{50}, IC_{70} or IC_{90} (one for each experiment) were considered as dependent variables in an analysis of variance (ANOVA) model where each experiment was considered as an independent replicate. Data in tables show the mean ± standard error (SE) of independent experiments. Differences between groups were assessed using the two tailed Student's *t*-test. The processing and statistical analysis of the data were performed by using the Statgraphics Plus 5.0 software package (Manugistics Inc., Rockville, MD).

3. Results

3.1. Gene marking of WEHI-3b cells with EGFP

Lentiviral vectors (LVs) carrying the EGFP (Enhanced Green Fluorescent Protein) gene (as a marker gene), under the control of the different promoters, were used for the transduction of WEHI-3b cells. The spleen focus-forming virus (SFFV) promoter was clearly the strongest with a Mean Fluorescence Intensity (MFI) of 210, although this LV was the least efficient transducing WEHI-3b cells (11.7% of $EGFP^+$ cells). The highest percentage of transduced cells was obtained with the cytomegalovirus (CMV)–LV (78.3% of $EGFP^+$

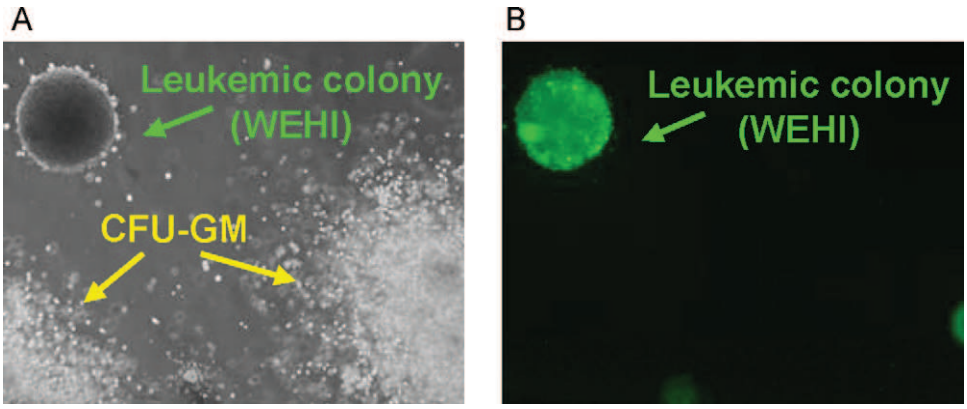

Fig. 1. Microphotographs of CFU-GM and transduced WEHI-3b colonies in semisolid cultures.

cells). Consequently, the CMV–LV was chosen for further experiments (Supplementary Fig. 1A–C).

3.2. Transduction does not affect to the characteristics of WEHI-3b cells

Proliferation rate was not affected by the CMV–LV transduction and the expression of EGFP, the replication time was 21 h for both transduced and parental cells. In the same way, the clonogenicity capacity did not show any change. In semisolid cultures, 25 transduced or parental cells produced 21 ± 9 and 22 ± 2 colonies, respectively.

In vivo leukemogenesis of transduced cells was also tested. Three weeks after de transplantation of 10^4 transduced WEHI cells with 2×10^6 healthy BMC into irradiated mice, leukemia symptoms were deduced from the appearance of ataxia of back legs, lethargy and cachexia. After an organ examination, splenomegaly, hepathomegaly were also detected. Histological studies evidenced infiltration of leukemic cells into various organs such as, lung, spleen and liver. The development of leukemia induced by transduced WEHI cells followed a pattern similar to the disease induced by the untransduced cells (Supplemented Fig. 2).

3.3. Leukemia and healthy hematopoietic cells can be distinguished in the same culture

Colonies of transduced WEHI-3b cells were distinguishable from healthy CFU-GM colonies of murine BM in semisolid cultures under fluorescence microscopy (Fig. 1). Furthermore, the clonogenicity of both types of colonies was not affected by the presence of each other. Mixed cultures were performed with 20, 40 or 60 WEHI-3b cells together with different amounts of murine BMC (5000, 10,000 and 20,000). The growth of WEHI-3b colonies was independent of the CFU-GM colonies in the same culture (Supplementary Table 1). Similarly, regardless of the number of leukemic cells seeded in the methylcellulose cultures, the clonogenicity of CFU-GM colonies did not vary. Cultures of only BMC showed 263 colonies per 10^5 seeded cells. When different amounts of WEHI-3b cells were also added in the same cultures, the number of CFU-GM colonies ranged from 265 to 272, without statistically significant differences.

Taking into account these results, it was chosen to perform mixed cultures with 10,000 BMC and 40 WEHI-3b cells for testing their utility in the study of sensitivity of antileukemic compounds.

3.4. In vitro treatment of leukemic cells impairs their leukemogenic capacity

Leukemic cells were treated *in vitro* for 24 h with a dose of daunorubicin which reduced the survival of WEHI-3b colonies to 10% ($IC_{90} = 173.87 \pm 7.65$ nM) and, then transplanted into lethally irradiated mice together with 2×10^5 healthy BMC as support in the hematopoietic syndrome post-irradiation (Supplementary Fig. 3). Animals receiving 100 and 1000 untreated WEHI-3b cells did not show disease symptoms, with survival rates of 100% and 80%, respectively. Mice transplanted with 10,000 untreated leukemic cells died because of the leukemia development. However, when 10,000 WEHI-3b cells previously treated with the IC_{90} of daunorubicin were transplanted, the survival of mice (100%) was similar to that reached in the group transplanted with 1000 untreated cells (80%).

3.5. Effect of antileukemic compounds on mixed cultures

Sensitivity of leukemic cells and healthy myeloid cells (CFU-GM) to the different compounds was studied both in standard and mixed clonogenic assays. Regarding daunorubicin and using the standard

Table 1
Inhibitory concentration (IC) values and therapeutic indexes (TI) of CFU-GM and WEHI-3b cells after exposure *in vitro* to different concentrations of daunorubicin (positive control), atropine sulphate (negative control) trabectedin, Zalypsis and PM01183 in the semisolid mixed cultures.

		IC_{50} (nM)	TI	IC_{70} (nM)	TI	IC_{90} (nM)	TI
Daunorubicin (nM)	CFU-GM	17.71 ± 1.32	2.74	22.88 ± 0.59	3.00	28.05 ± 0.99	3.19
	WEHI colonies	6.45 ± 0.20*		7.61 ± 0.19*		8.77 ± 0.37*	
Atropine sulphate (mM)	CFU-GM	0.33 ± 0.05	0.94	0.47 ± 0.06	1.02	0.61 ± 0.09	1.09
	WEHI colonies	0.35 ± 0.08		0.46 ± 0.08		0.56 ± 0.08	
Trabectedin (nM)	CFU-GM	0.48 ± 0.07	1.77	0.66 ± 0.08	2.06	0.83 ± 0.10	2.12
	WEHI colonies	0.27 ± 0.04*		0.32 ± 0.03*		0.39 ± 0.02*	
Zalypsis® (nM)	CFU-GM	4.99 ± 0.45	1.85	6.70 ± 0.51	1.87	8.38 ± 0.58	1.87
	WEHI colonies	2.69 ± 0.28*		3.58 ± 0.32*		4.48 ± 0.39*	
PM01183 (nM)	CFU-GM	0.66 ± 0.09	1.53	0.84 ± 0.09	1.55	1.03 ± 0.10	1.58
	WEHI colonies	0.43 ± 0.03*		0.54 ± 0.02*		0.65 ± 0.01*	

TI = CFU-GM IC(50, 70 or 90)/WEHI IC(50, 70 or 90).
* $p < 0.05$ WEHI colonies vs. CFU-GM.

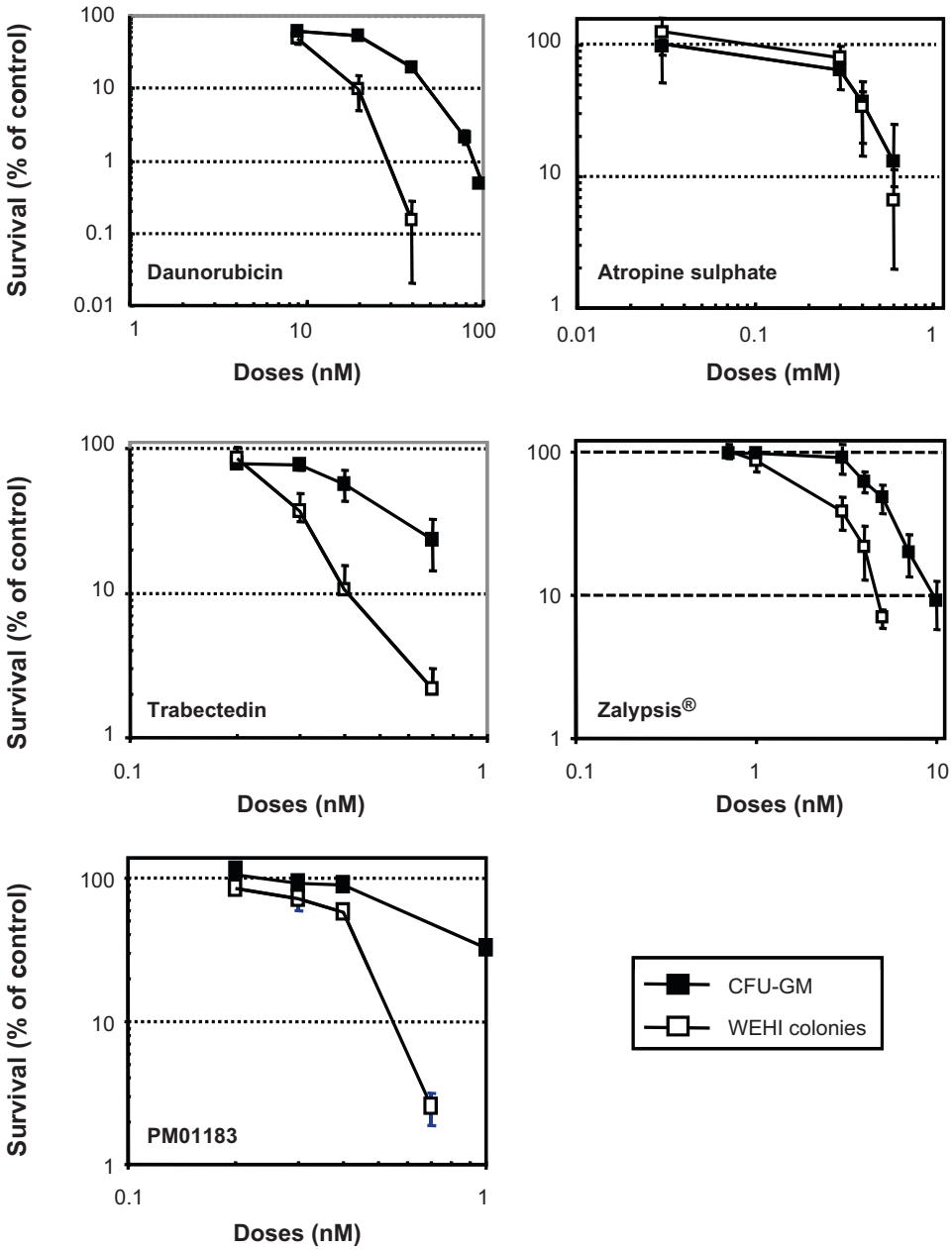

Fig. 2. Survival curves of CFU-GM and WEHI-3b colonies in mixed cultures.

clonogenic assays, IC values for WEHI-3b cells (IC_{50}: 6.43 nM) were significantly lower that the values for CFU-GM progenitors (IC_{50}: 19.36 nM) (Supplementary Table 2). The same was true when the mixed cultures were used (Table 1, Fig. 2). In the case of atropine sulphate, the effect was the same both on leukemic and healthy cells regardless the kind of clonogenic culture used and there were not significant differences between the sensitivity of WEHI and CFU-GM colonies.

As far as trabectedin and Zalypsis® were concerned, both compounds showed a differential effect on leukemic and healthy myeloid progenitors. Trabectedin was 1.77-fold more effective on WEHI cells than in CFU-GM progenitors. This difference of sensitivity was of 1.85-fold in the case of Zalypsis®. There were no statistically significative differences between the IC values from the classical and the mixed cultures. Similar results were obtained when a new antitumoral compound in preclinical development, PM01183, was used. These results also showed a higher cytotoxic effect on leukemic cells compared to the healthy CFU-GM progenitors with a therapeutic index (TI) of 1.55.

4. Discussion

Drug development is a costly process both because of time reasons and financial reasons. A large proportion of these compounds (approximately 90%), fail due to toxicity (Davies et al., 2008). To decrease costs in the drug discovery process, the pharmaceutical industry has invested in high throughput automated technologies which has brought a lot of advantages. However, in the last years, there has been an increasing demand of new cell based assays (Brinker and Caldwell, 2008). Taking into account that the main target organs involved in the development of toxicity are the liver, the skin and the BM, *in vitro* systems capable of predicting these organ toxicities would be invaluable in the preclinical stages (Nassar et al., 2004). The necessity of developing assays for the study of selec-

tivity of antitumoral compounds in parallel with its toxic effect has already been pointed out some years ago (D'Incalci, 2002). In this sense, our study is focused on the development of a cellular system including both leukemic and healthy hematopoietic cells for the study of efficacy and toxicity of new antileukemic compounds all at once, which could provide complementary and important information for a safe conduction of clinical trials.

For this purpose, two cellular populations have been used: myeloid progenitor cells (CFU-GM) from murine BM, as healthy cells, and, WEHI cells as a leukemic population. The CFU-GM assay has been proved as a useful tool in the prediction of toxic effects of antitumoral compounds in the myeloid compartment of the hematopoietic system, which is responsible for the formation of granulocytes and monocytes (Albella et al., 2007). As a consequence of a possible toxic effect of antitumoral compounds on this compartment, neutropenias could be developed and lead to risky situations to the patient. On the other hand, since it was described in 1969, WEHI-3b cell line has been extensively used as a model of AML (Warner et al., 1969) providing with valuable results during four decades of research. In our study, WEHI cells were marked with the EGFP gene by means of a LV with the intention to make them easily distinguishable from the healthy cells in a mixed culture.

There are several interests in the transduction of AML cell lines or primary leukemic cells with LVs. Among others are the *in vivo* follow up of leukemic cells transplanted in mice, introduction of suicide genes or specific antigen expression for the development of vaccines leading to antileukemic immunity. WEHI-3b cells have been previously transduced with retroviral (Garcia-Castro et al., 2000), adenoviral (Garcia-Castro et al., 2001) and LVs (Ling et al., 2006) carrying different reporter genes under different promoters. Proliferation rate and clonogenicity of transduced leukemic cells did not show significant differences when compared to untransduced cells. Transplantation of transduced WEHI cells lead to the development of leukemias with similar characteristics to the disease produced for the parental cells and also reported in previous studies (Garcia-Castro et al., 2000; Ling et al., 2006) Although EGFP has been previously used as a marker in leukemic cells with other purposes, to the best of our knowledge, this is the first time that a transduced leukemic cell line is used for being distinguishable from healthy hematopoietic cells in *in vitro* mixed clonogenic cultures, making possible the scoring of both types of colonies in the same culture.

Although the outcome of patients with AML has improved as a result of intensive cytarabine- and anthracycline-based chemotherapy, AML remains a difficult disease to treat, especially in adult patients (Burnett, 2002). The challenge is to find other strategies focusing on new drugs with new mechanisms of action compared to conventional chemotherapy (Möllgård et al., 2008; Nahi et al., 2008). The anthracycline daunorubicin is one of the major and well-established therapeutic agents widely used in the treatment of AML (Laurett and Jaffrezou, 2001) and, because of that, this compound has been used as a positive control in the development of the mixed culture of our study. As it was expected, the leukemic cells were more sensitive to daunorubicin than the healthy population. Furthermore, the capacity of the WEHI cells, previously treated *in vitro* with a dose reducing their growth by a 90% (IC_{90}), for developing leukemia *in vivo* was decreased according with the *in vitro* treatment. Mice transplanted with 10,000 untreated leukemic cells died because of the leukemia development. However, when 10,000 WEHI-3b cells previously treated with the IC_{90} of daunorubicin were transplanted, the survival of mice (100%) was similar to that reached in the group transplanted with 1000 untreated cells (80%). These results showed that the *in vitro* damage of WEHI cells was reflected in their *in vivo* decreased capacity for developing leukemia.

TI calculated from the data obtained with the standard cultures showed that daunorubicin was about 3 times more effective on WEHI cells than on CFU-GM, and this value was not significantly different in mixed cultures. Atropine sulphate was used as a negative control because this drug is not an antitumoral compound. It is used in ophthalmology, before eye examinations to open the pupil, as well as to relieve pain caused by swelling and inflammation of the eye. It is also used as an emergency antidote in organophosphate poisoning, as well as in mushroom poisoning. Therapeutic blood concentrations are in the range of 0.05–0.3 nM. Our results showed effect on murine hematopoietic progenitors in the range of mM which is coincident with the sensibility of human myeloid progenitors (Cerrato et al., 2009). These results agree with the lack of hematological side effect after administration of this pharmaceutical in humans. As it was expected, atropine sulphate did not show any differential effect between leukemic and healthy colonies neither in standard nor mixed cultures.

In order to test the usefulness of the assay described in this study, two new antitumoral compounds were tested trabectedin (Yondelis®) and Zalypsis®. Both of them are antitumor agents of marine origin. Trabectedin is approved by EMEA for the treatment of advanced or metastatic soft sarcoma (Cuevas and Francesch, 2009) and, in combination with liposomal doxorubicin, for the treatment of patient with relapsed platinum-sensitive ovarian cancer. It has a unique action mechanism, binding to the minor groove of the DNA and interferes with cell division and the gene transcription processes and repair machinery of the DNA (Herrero et al., 2006; Soares et al., 2007). The dose limiting toxicity (DLT) is hematological and it was previously described its effect on murine and human hematopoietic progenitor and stem cells (Albella et al., 2002; Gomez et al., 2003). Our results showed that this compound has a higher effect on leukemic cells compared with CFU-GM from murine BM, with a TI of about 2. Compared to trabectedin, Zalypsis® showed a similar TI, however its effect on normal hematopoietic progenitors are about 10-fold lower. There are not still published data about the DLT of this new compound but our results would agree with the lack of toxicity observed in xenograft murine models of human cancer. In those studies a significant tumor growth inhibition was demonstrated and the tumoral cell lines used presented *in vitro* IC_{50} values in the mid-nanomolar range (Leal et al., 2009; Ocio et al., 2009). The differences in the mechanism of action between trabectedin and Zalypsis® may account for the different effect on hematopoietic progenitors.

There were no statistically significative differences between the IC values from the standard cultures compared to mixed clonogenic assays. Taken together, our results showed that the growth of leukemic and healthy BM colonies can be assayed in the same clonogenic culture without interfering in their individual properties. The mixed culture developed in this study was applied to a novel antitumoral compound in preclinical development. Our results showed that PM01183 also exerts a higher effect on leukemic cells compared to healthy myeloid progenitors (TI:1.5). As far as the hematotoxicity is concerned, the effect of PM01183 on healthy myeloid progenitors is similar to the effect exerted by trabectedin, with IC_{50} values of 0.66 and 0.43 respectively. Taken into account that the DLT of trabectedin is hematological, caution should be taken with the hematotoxicity when PM01183 is *in vivo* administered.

All together, our results indicate that the mixed culture developed in this study could be used to define the antileukemic effect of new antitumoral compounds in early stages of development. Furthermore, all the new antitumoral compounds studied exert antileukemic myeloid activity *in vitro* which deserves further *in vivo* investigations. In order to prove the potential of this assay to predict the antileukemic effect of new compounds, it should be formally validated prior to be applied (Balls, 1995a,b, 2010). However, the

development of antitumoral compounds could take advantage of strategies like the one described in this study, saving time and facilitating the reduction of the number of animals used in preclinical development of pharmaceuticals.

Conflict of interest statement

Sandra Martínez: PharmaMar employee. The other authors reported no potential conflicts of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.toxlet.2010.09.014.

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Gene Therapy (2010), 1–10

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ORIGINAL ARTICLE

Immunoresponse against the transgene limits hematopoietic engraftment of mice transplanted *in utero* with virally transduced fetal liver

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In utero cell and gene therapies constitute alternative strategies to the postnatal treatment of inherited diseases. Fetal hematopoietic progenitors could be a potential source of donor cells for these strategies. In this study, hematopoietic lineage-negative fetal liver cells from 14.5-day-old fetuses were transduced under different cytokine and culture combinations using a lentiviral vector expressing the enhanced green fluorescent protein (EGFP). When cells were transduced for 6 h in the presence of mSCF, hTPO and FLT3-L in retronectin-coated dishes at a multiplicity of infection of 10 transduction units/cell, up to 70% of granulo–macrophage colony-forming cells expressed the *EGFP* reporter gene. *In utero* transplantation experiments revealed that conditions leading to high transduction efficiencies were associated with poor engraftments of syngeneic recipients. Significantly, this effect was associated with the detection of a humoral and cellular immunoresponse against the transgenic protein. Moreover, the humoral response against EGFP was detected not only in *in utero* transplanted recipients but also in the operated mothers, suggesting the maternal origin of the anti-EGFP immunoresponse. These observations reinforce the necessity of carefully studying the potential immunoresponses in future prenatal gene therapy protocols.
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Keywords: *in utero* transplantation; fetal liver; hematopoietic stem cell; transgene immunoresponse

INTRODUCTION

Hematopoietic transplantation is the therapy of choice for a wide range of blood disorders in adults.[1] However, for the successful engraftment of exogenous healthy cells, this type of transplant requires a compatible donor and myelosuppressive treatment, and major complications may arise, such as graft-versus-host disease and graft rejection, compromising both the success of the treatment and patient survival.[2] Further, in some inherited diseases, hematopoietic transplantation after birth or in adulthood is not always effective because the disease may have already had an effect during fetal development.[3,4] Advances in genetic testing and the possibility of sampling chorionic villus cells during the first trimester of gestation facilitate early prenatal diagnosis.[5] These technical improvements permit the treatment of a fetus during a theoretical window of opportunity from the last weeks of the first trimester to the second trimester in humans, in which the early gestational immune system is undergoing a process of self-education. Thus, *in utero* hematopoietic cell transplantation (IUHCT) could provide the appropriate conditions for specific tolerance to allogeneic cells or to genetically corrected autologous cells.[6,7] In effect, *in utero* transplantation of hematopoietic stem cells (HSCs) has been used to treat inherited diseases over the past 30 years. First attempts were performed in mice and sheep, and significant levels of chimerism achieved without myeloablative treatment. *In utero* transplantation in human fetuses has been successful only in immunodeficient patients.[8] Evidence of *in utero* engraftment of allogeneic cells has been reported in different animal models and in humans.[9] However, the recently described failure of long-term chimerism after IUHCT of allogeneic grafts in mouse models[10] suggests the immunocompetent status of the fetus. Thus, the use of autologous, genetically corrected cells for transplant could avoid potential problems associated with the rejection of allogeneic cells by the recipient. In a previous study, we reported the long-term, multilineage engraftment of retrovirally transduced adult bone marrow (BM) HSC after IUHCT of syngeneic mouse fetuses.[7] These models of *ex vivo* gene therapy avoid vector infusion in the animals, thus limiting the transduction of non-target cells (that is, germline) and reducing the risk of immunoreaction with the transgene.[4,11]

Although the principal sources of hematopoietic cells for transplantation in humans are adult BM, mobilized peripheral blood and umbilical cord blood, fetal liver (FL) hematopoietic progenitors are a good alternative for IUHCT because both recipient and graft coincide in developmental stage.[8] In addition, the expression profiles of adhesion molecules in FL-HSCs (FL-HSCs)[12] could confer cells improved homing and engraftment capacities over adult HSCs in response to IUHCT. In a gene therapy context, recent studies have shown that FL-HSCs proliferate more actively than adult BM HSCs[13,14] and are differentially regulated by transcription factors.[15] These findings suggest that specific experimental conditions may be required for the transduction of FL-HSCs with integrative vectors. Some success has already been described for the transplant of

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genetically corrected FL-HSCs in newborn mice.[16] However, as far as we are aware, the possibility of engrafting mice after the *in utero* transplant of FL-HSCs transduced with integrative vectors has not yet been reported.

In this study, we optimized the conditions required to transduce FL hematopoietic progenitors using lentiviral vectors (LVs) and examined the long-term engraftment capacity of these lentivirally transduced FL-HSCs following IUHCT. Our findings unveil the existence of humoral and cellular immunoresponses against the transgenic protein after *in utero* transplantation.

RESULTS

Efficient lentiviral transduction of hematopoietic FL progenitors in the presence of SCF, hTPO, FLT3-L and retronectin

To improve the lentiviral transduction of FL-HSCs, 5×10^5 lineage-negative (Lin^-) FL cells from 14.5-day-old mouse fetuses (immunophenotyping of this population is showed in Supplementary Figure S1) were transduced for different times at multiplicities of infection (MOI) of 5 and 10 TUs per cell, and different combinations of growth factors (mSCF+hIL-11 versus mSCF+hTPO+FLT3-L), both in the presence and the absence of retronectin. After transduction, cells were washed and cultured in semisolid media to analyze the contents and transduction efficiencies of committed progenitors (Figure 1). To minimize cell manipulation and bearing in mind that FL progenitors are actively proliferating, cells were not prestimulated but were transduced for very short incubation periods. As shown in Figure 1a, highest transduction levels (up to 70% of CFU-GM positive for enhanced green fluorescent protein (EGFP)) were recorded after 6 h of transduction (MOI 5 or 10) using mSCF+hTPO+FLT3-L in the presence of retronectin. The comparison of the number of colonies generated from mock-transduced samples and samples transduced with the LVs indicates that the LV transduction did not affect the viability of the progenitors present in the samples (Figure 1b). Transduction periods longer than 6 h affected the viability of FL progenitors (data not shown). Incubating the Lin^- FL cells in the presence of retronectin increased both the percentage of transduced CFU-GM and the total number of colonies, regardless of the culture conditions used (Figure 1b).

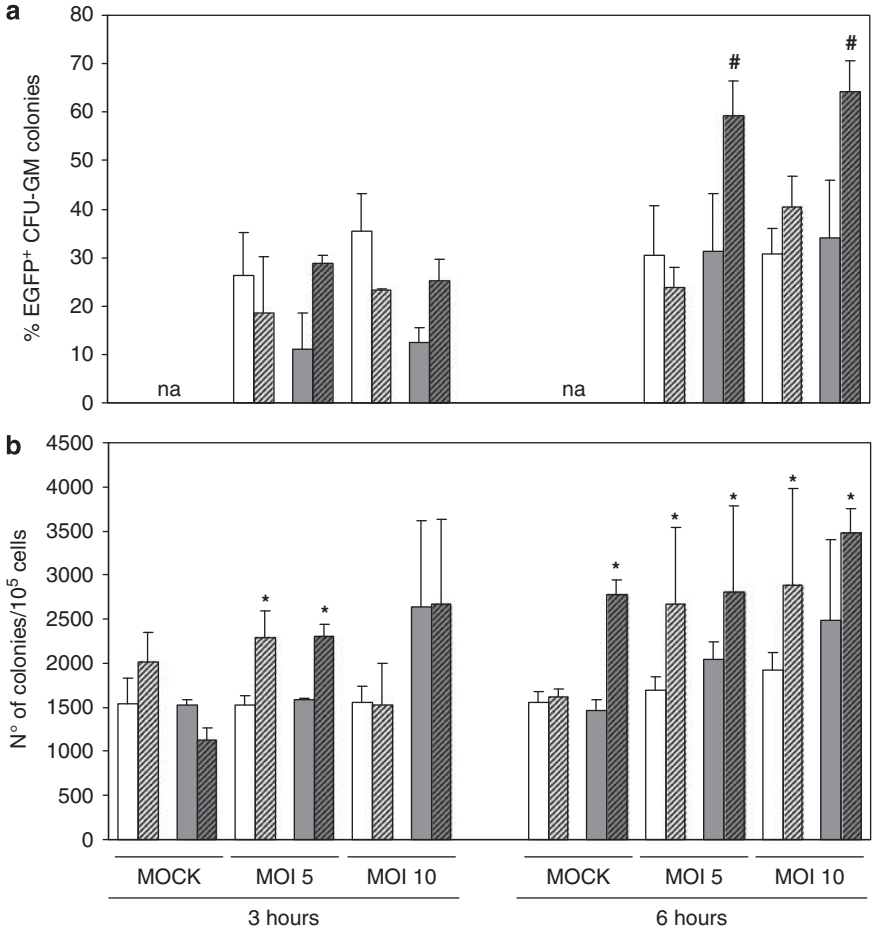

Figure 1 Optimizing the *in vitro* conditions for the transduction of FL hematopoietic progenitors using LVs. Lin^- FL hematopoietic cells from 14.5-day-old fetuses were mock transduced or transduced with EGFP-LVs for 3 and 6 h at MOIs of 5 and 10 TUs/cell using different combinations of hematopoietic growth factors, either in the presence or absence of retronectin. After transduction, the cells were washed and cultured in semisolid media to assess the growth of granulo–macrophage progenitors (CFU-GM). (**a**) Percentage of $EGFP^+$ colonies with respect to the total number of colonies recorded for each infection condition. (**b**) Total number of colonies scored; (□) hIL-11 plus mSCF, (■) mSCF plus hTPO plus FLT3-L; hatched bars indicate the same combinations of cytokines in the presence of retronectin; #Statistically significant with respect to the remaining groups; NA, not applicable; *Statistically significant with respect to the same conditions tested in the absence of retronectin.

Lentivirally transduced FL progenitors are capable of long-term engraftment in myeloablated adult recipients

To assess the engraftment ability of transduced FL progenitors, cells were incubated for 6 h with LVs (MOI 10) in the presence of mSCF+IL (interleukin)-11 or mSCF+FLT3-L+hTPO with or without retronectin, and then infused into adult irradiated congenic mice. In all, 2–3×10^5 Lin$^-$ transduced viable donor cells (CD45.1) were transplanted per recipient (CD45.2) and the level of donor hematopoiesis followed by flow cytometry. Most hematopoiesis of primary transplanted recipients was of donor origin, independently of the culture conditions (Figure 2 and Supplementary Figure S2). No specific mortality was observed in any of the transplant groups (not shown), indicating sufficient numbers of progenitors/HSCs were transplanted after the transduction protocol, in good consistency with data obtained in the clonogenic studies of Figure 1b.

A significant proportion of these cells (up to 45%) expressed the *EGFP* transgene (Figure 2c). As for the CFU-GMs, the group transplanted with cells that had been transduced in the presence of mSCF, FLT3-L, hTPO and retronectin showed the highest levels of EGFP-positive peripheral blood cells (Figure 2c). An average of 30% EGFP-expressing cells was achieved 90 days after transplant. BM analyses performed at 180 days after transplant, before secondary transplants (see below), suggested the stability of the transgene expression in the long term (Figure 3).

To confirm the long-term engraftment of transduced FL-HSCs, 5×10^6 BM cells from primary recipients were transplanted into secondary myeloablated recipients (Figure 3). Two animals per group were selected as donors for the secondary transplant. In all the experimental groups, secondary recipients were completely engrafted with FL-HSC-derived cells and the percentage of engraftment was maintained up until 60 days after transplantation (Figure 3a). Moreover, these mice showed similar or even higher percentages of EGFP-expressing cells in peripheral blood compared with primary recipients (Figure 3b), confirming the expression stability of the marker gene in very primitive HSCs.

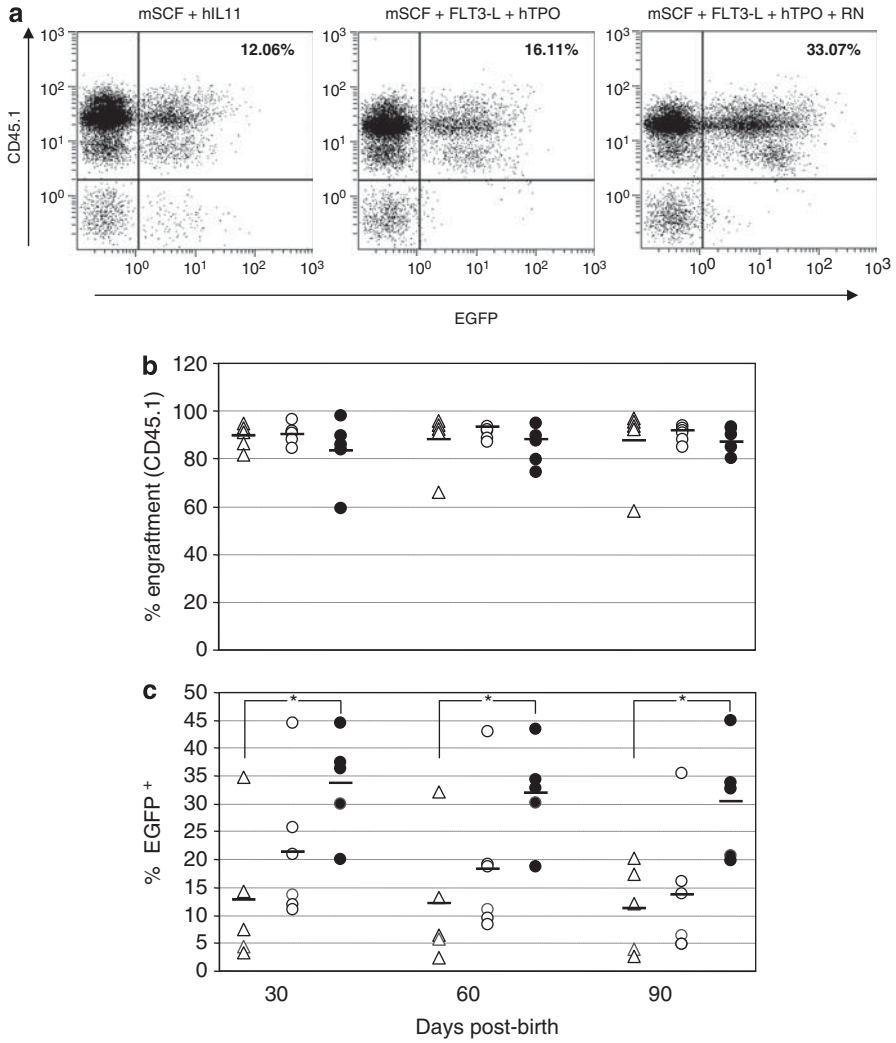

Figure 2 Long-term engraftment of lentivirally transduced FL cells in adult recipients. FL Lin$^-$ cells (CD45.1) were transduced for 6 h using LVs (MOI of 10 TUs/cell) in the presence of hIL-11 and mSCF (Δ) or mSCF, hTPO and FLT3-L without (○) or with (●) retronectin. Transduced cells were then transplanted into lethally irradiated adult recipients (CD45.2), and the engraftment of transduced donor cells was monitored over time. (**a**) Representative flow cytometry analysis of the engraftment of transduced (EGFP+) and non-transduced (EGFP−) exogenous (CD45.1$^+$) cells after 30 days of transplantation; (**b**) individual engraftment levels in recipient mice; and (**c**) analysis of the percentage of hematopoietic engrafted cells expressing EGFP. Each symbol represents a single mouse; and horizontal lines represent the median recorded in each group. *Statistically significant ($P<0.05$).

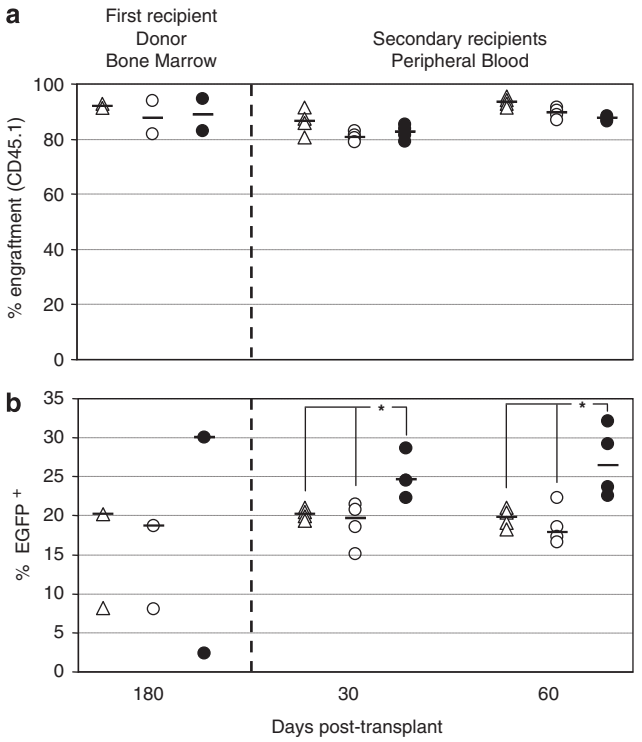

Figure 3 Engrafting secondary recipients with BM samples from primary recipients transplanted with LV-transduced FL cells. BM cells from adult animals transplanted with transduced FL progenitors were retransplanted into lethally irradiated secondary recipients. Percentages of donor cells (%CD45.1) and percentages of donor cells expressing EGFP (% of EGFP$^+$ in the CD45$^+$ population) in the BM of primary recipients before transplant into secondary recipients are shown on the left side. Data corresponding to secondary recipients are shown on the right side: (**a**) percentage of engraftment; (**b**) percentage of hematopoietic-engrafted cells expressing EGFP; symbol details as in Figure 2; each symbol represents a single mouse; and horizontal line represents the median recorded in each group. *Statistically significant with respect to the remaining groups.

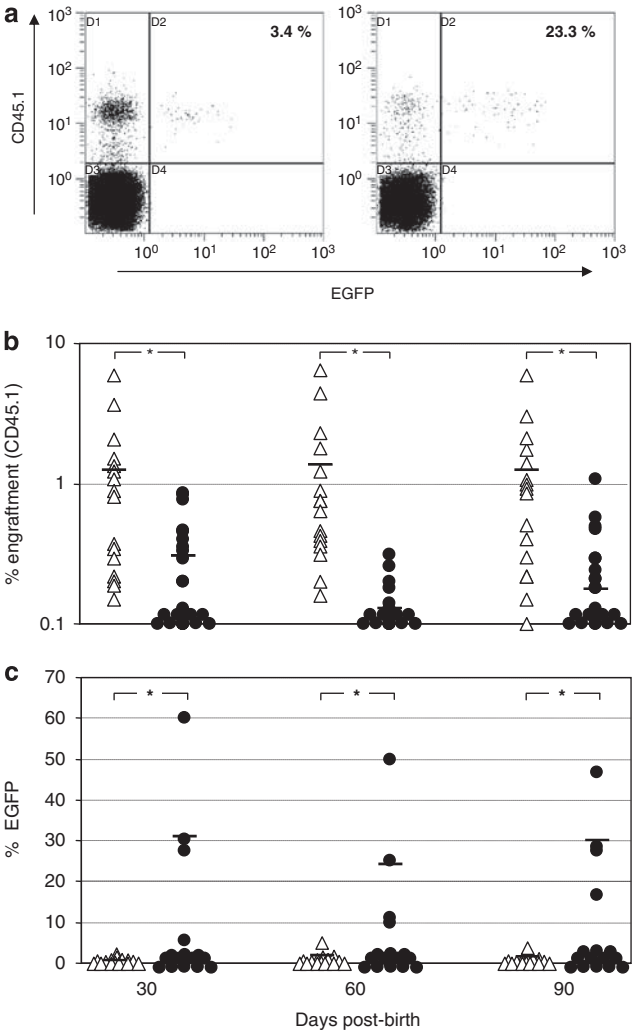

Figure 4 Engraftment and EGFP expression after the *in utero* transplant of transduced Lin$^-$ FL cells. Lin$^-$ FL cells from CD45.1$^+$ mice were transduced for 6 h with EGFP-LVs (MOI 10 TUs/cell) and then transplanted *in utero* into 14.5-day-old fetuses (CD45.2). The engraftment process was followed until 90 days after birth. (**a**) Representative flow cytometry analysis of peripheral blood obtained after 30 days of the *in utero* transplantation of two representative mice with transduced Lin$^-$ cells in the presence of mSCF and hIL-11 (left panel), or in the presence of mSCF, FLT3-L, hTPO and retronectin (right panel), respectively; (**b**) percentage engraftment in recipient mice; (**c**) percentage of hematopoietic-engrafted cells expressing EGFP; (Δ) mice engrafted with cells transduced with hIL-11 and mSCF; and (●) mice engrafted with cells transduced with mSCF, hTPO and FLT3-L in the presence of retronectin. Each symbol represents a single mouse. Horizontal line represents the median recorded in each group. *Statistically significant ($P<0.05$).

Engraftment of lentivirally transduced Lin$^-$ FL cells in fetal mice

In utero transplantation experiments were performed as two different experimental groups. The first group of fetuses was transplanted with FL progenitors transduced in the presence of mSCF and hIL-11, which conferred moderate transduction efficiencies. The second group was transplanted with FL progenitors transduced in the presence of mSCF, FLT3-L and hTPO in retronectin pretreated plates, which allowed a higher transduction efficacy of FL progenitors and HSCs. In both groups, cells were infected for 6 h at a MOI of 10 TUs per cell. As control, an *in utero* transplantation (IUT) with non-manipulated cells was also performed (Supplementary Table S1).

To check for the presence of donor cells in the *in utero* transplant recipients, donor engraftment levels were determined up until one year after transplant. Representative dot-plot analyses are shown in Figure 4a. Results obtained at 30, 60 and 90 days after birth are represented in Figures 4b and c. When the IL-11/SCF combination was used for transduction, 68% of the animals born showed detectable ($>0.1\%$) stable engraftment of donor cells. Chimerism levels in this group were similar to those observed in mice *in utero* transplanted with non-manipulated cells. However, the percentage of engrafted mice was significantly lower (35%) when the FL3/TPO/SCF/RN combination was used (see Supplementary Table S1). Also, in this second group, donor engraftment rates were significantly lower (around 0.2 versus 2% in the former group; Figure 4b). However, the percentage of EGFP$^+$ cells in the positive mice was much higher when the FL3/TPO/SCF/RN combination of growth factors was used (up to 60% of engrafted cells were EGFP$^+$) (Figure 4c).

Immunoresponse against the exogenous transgenic protein in mice transplanted *in utero* with transduced FL cells

To determine whether mice transplanted *in utero* with transduced FL progenitors generate an immunoresponse against the transduced cells,

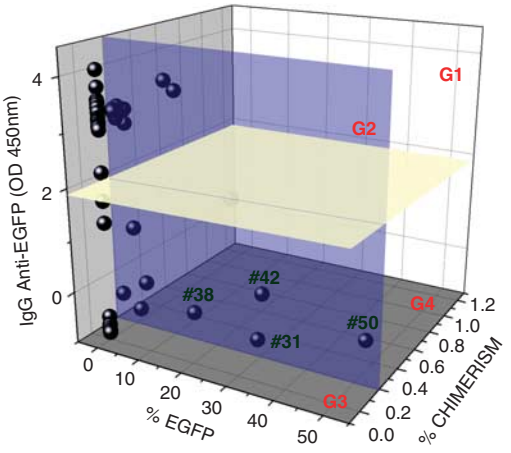

Figure 5 Determining anti-EGFP antibodies in mice transplanted *in utero* with LV-transduced FL cells. After 150 days of birth, serum from mice transplanted with FL progenitors transduced with EGFP-LVs (6 h; MOI 10 TUs/cell; mSCF, FLT3-L, hTPO and retronectin) was collected and assayed for humoral immunoresponses against EGFP by enzyme-linked immunosorbent assay. Each point represents a single mouse. Colored squares classify the mice into four groups: G1, engrafted mice with no EGFP$^+$ cells showing anti-EGFP antibodies; G2, non-engrafted mice showing anti-EGFP antibodies; G3, non-engrafted animals showing anti-EGFP antibodies; and G4, engrafted animals showing no anti-EGFP antibodies. Green numbers indicate mice showing EGFP$^+$ cells.

we examined the presence of anti-EGFP antibodies in the serum of recipients transplanted with cells transduced in the presence of mSCF+FLT3-L+hTPO (data obtained at 5 months after birth). A total of 31 out of 51 animals showed specific antibody responses against the exogenous protein. None of the mice showing anti-EGFP antibodies presented EGFP-expressing cells, despite the fact that 26% of these animals had been long-term engrafted with exogenous FL cells. According to chimerism and the presence of anti-EGFP antibodies, we were able to establish four different groups of recipients (Figure 5): mice engrafted with exogenous cells without anti-EGFP antibodies and with EGFP-expressing cells (region G4); mice engrafted with exogenous cells with anti-EGFP antibodies and without EGFP-expressing cells (region G1); and non-engrafted mice that were positive (region G2) or negative (region G3) for anti-EGFP antibodies. Remarkably, animals with EGFP$^+$ cells (4 out of 51 with 6–60% of EGFP$^+$ long-term engrafted cells) were only observed in the group that showed no antibodies against the transgenic protein (Figure 5).

To examine the type of specific humoral immunoresponse produced in these animals, nine mice were injected with 2 μg of recombinant EGFP at 5 months after birth. Serum was collected every 2 days after the injection and immunoglobulins G (IgGs) and immunoglobulins M (IgMs) against EGFP determined by enzyme-linked immunosorbent assay (Figures 6a and b, respectively). Significantly, animals that showed a secondary response to EGFP were always negative for EGFP$^+$ cells.

Cell responses to EGFP were assessed in 12 *in utero* transplanted mice by inducing EGFP-specific proliferation and INFγ release by activated cells. Non-engrafted animals as well as animals engrafted with EGFP$^-$ exogenous cells showed cell responses against EGFP (Figures 6c and d). Collectively, these data point to a complete immunoresponse elicited by the EGFP that impairs engraftment and eliminates EGFP-expressing cells.

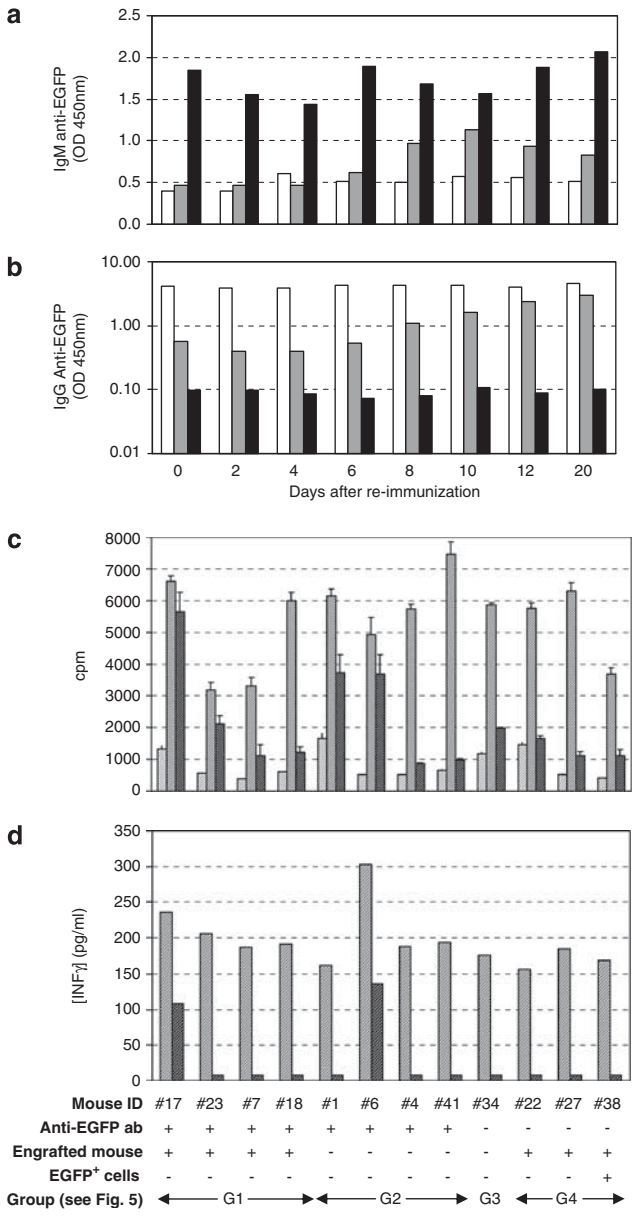

Figure 6 Humoral and cell immunoresponses against EGFP in mice transplanted *in utero* with LV-transduced FL cells. The humoral immunoresponse was examined in mice reimmunized with recombinant EGFP after 150 days of birth. Serum samples from the animals were collected every second day for 20 days; (**a**) IgM determined by enzyme-linked immunosorbent assay; and (**b**) IgG determined by enzyme-linked immunosorbent assay. Three representative mice are shown: (□, ▨) two non-engrafted mice showing secondary responses to EGFP; and (■) mouse engrafted with EGFP$^+$ cells not showing a secondary response to EGFP. The cell immunoresponse was assessed 6 months after birth. (**c**) Cell proliferation in response to EGFP determined by H^3-thymidine incorporation. (□) Splenocytes cultured alone; (▨) splenocytes cultured in the presence of PHA; (▨) splenocytes cultured in the presence of EGFP. (**d**) Interferon-γ (INFγ) release measured by specific enzyme-linked immunosorbent assay. (▨) Supernatants from splenocytes cultured in the presence of PHA; and (▨) supernatants from splenocytes cultured in the presence of EGFP.

Transgene silencing in lentivirally transduced and *in utero* transplanted FL cells

To determine whether, besides the specific immunoresponse, transgene silencing was taking place in animals transplanted *in utero* with

transduced cells, the presence of EGFP-LV proviruses in animals showing exogenous engraftment was analyzed by PCR in peripheral blood cells 1 year after birth. All the animals analyzed, scoring either positive or negative for EGFP expression, showed the presence of the EGFP provirus, indicating silencing of the EGFP transgene after *in utero* transplantation. Interestingly, we noted that mice with EGFP$^+$ cells presented a viral copy number that was inversely proportional to the percentage of EGFP$^+$ cells (Figures 7a and b).

The maternal immunological response against the transgene impairs the hematopoietic chimerism in recipient mice after *in utero* transplantation of lentivirally transduced cells

To investigate whether any leakage of donor cells and/or reabsorption of donor cells within aborted fetuses could induce an immunoresponse of the injected mothers against the transgene, serum samples extracted from the mothers at 2 days after surgery and at 2 days postpartum were analyzed for the presence of anti-EGFP antibodies. Additionally, serum samples from the pups at 2 days after transplant (17.5-day-old fetuses) and at 2 days after birth were also analyzed. We investigated a primary immunoreaction by means of IgM production. As shown in Figure 7c, although no IgM anti-EGFP antibodies were observed in the pups, significant levels of anti-EGFP IgM antibodies were recorded in dams undergoing the intrauterine administration of EGFP$^+$LV-transduced cells. No significant levels of anti-EGFP IgG antibodies were detected at this time because of the very short time period between antigen exposure and serum IgG evaluation (data not shown). To further check maternal transfer of anti-EGFP immunoresponse, *in utero* transplanted pups with lentivirally transduced cells were transferred to foster mothers upon delivery. In no instance, significant IgG antibodies were detected in pups raised with foster mothers, whereas in two out of five pups raised with operated mothers, anti-EGFP IgG antibodies were detected at 4 weeks after birth (Supplementary Figure S3).

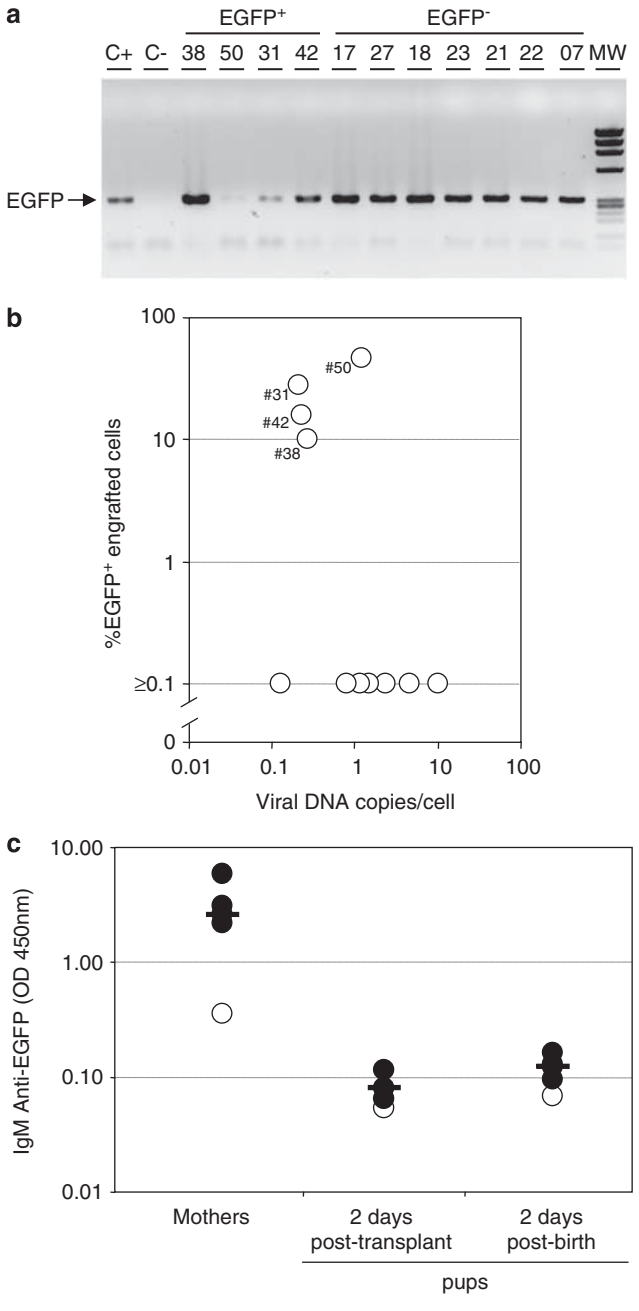

Figure 7 Transgene silencing and maternal response against the transgene in animals transplanted *in utero* with LV-transduced FL cells. (**a**) PCR of EGFP DNA sequences in genomic DNA extracted from the blood of *in utero* transplanted animals displaying significant exogenous engraftment after 1 year of birth. EGFP$^+$: mice with EGFP expressing cells as deduced from flow cytometry analysis; EGFP$^-$: mice undergoing exogenous engraftment but lacking EGFP-expressing cells; C+: DNA from an EGFP transgenic mouse; and C−: no DNA added to the PCR reaction mixture. MW, DNA molecular weight marker IX (Roche, Mannheim, Germany). (**b**) Correlation between integrated viral DNA copies per cell as determined by quantitative PCR and the percentage of EGFP$^+$ within the exogenous-engrafted cells in the blood of *in utero* transplanted animals after 1 year of birth. Numbers represent the identification of each mouse. (**c**) Humoral immunoresponse measured by enzyme-linked immunosorbent assay for the detection of anti-EGFP antibodies in the serum of operated mothers after 2 days of surgery and two days postpartum, and of pups after 2 days of transplant (17.5-day-old fetuses) and after 2 days of birth. Black dots, animals treated with EGFP-expressing LVs. Empty dots, serum pools from age-matched control animals not exposed to the EGFP. Each dot represents the analysis of a single animal. Solid line, mean values recorded for animals treated with EGFP-expressing LVs. Values 2-times over negative controls were considered positive.

DISCUSSION

This study was designed to test the engraftment capacity of genetically marked FL hematopoietic progenitors after their transplantation into syngeneic 14.5-day-old fetuses. As FL cells show different characteristics to adult BM cells, both in terms of their viral transduction and hematopoietic repopulation properties,[17] we explored specific *ex vivo* manipulation protocols capable of transducing FL progenitor cells. The highly proliferative stage of FL-HSCs[13] allowed us to shorten the lentiviral transduction procedure, minimizing the manipulation steps (that is, prestimulation time) that could compromise the repopulation capacity of FL-HSCs. The percentages of EGFP$^+$ progenitors obtained after transduction protocols of 3 h were not significantly affected by the transduction conditions used in our experiments. Transductions of 6 h were more reproducible and efficient, and revealed a benefit of using retronectin-pretreated plates while manipulating the samples. When compared with mock-transduced cells, the transduction did not affect the colony-forming ability of FL progenitors. We used VSV-G-pseudotyped LVs, which do not attach to the CH-296 fragment of fibronectin (retronectin).[18] However, our results are consistent with

previous observations showing that retronectin improves *in vitro* cell survival and gene transfer, independently of viral tropism.[19]

Several groups including ours have described that the combination of hIL-11 and mSCF maintains the BM HSC compartment in culture[20,21] and facilitates the transduction of adult hematopoietic cells using viral vectors. However, we did not observe similar effects herein using FL cells, and low transduction percentages were obtained when this combination of growth factors was used. These data are consistent with those of Bowie *et al.* who observed an inhibitory effect of hIL-11 on the self-renewal capacity of FL-HSCs cells.[22] In contrast, we obtained EGFP$^+$ progenitor proportions in excess of 59%, while preserving the total number of CFU-GMs, using the combination mSCF, FLT3-L plus hTPO during the transduction period. This combination of factors has been widely used for culture maintenance and transduction of umbilical cord blood hematopoietic progenitors.[23,24] However, as far as we know, this is the first report of the efficacy of this combination of cytokines when used for the transduction of mouse FL hematopoietic progenitors.

Our *in vivo* assays revealed the repopulating capacity of transduced FL cells after transplantation into irradiated adult mice, irrespective of the combination of cytokines used during the transduction procedure (Figure 2 and Supplementary Figure S2). However, the percentage of transduced cells recorded in recipient animals was always higher when the mSCF, FLT3-L and hTPO combination was used. Additionally, the percentage of EGFP$^+$ cells was kept stable until 90 days after transplant in primary recipients and for 60 days after transplant in secondary recipients (the last observation period). Altogether, our data indicate that FL repopulating hematopoietic cells can be efficiently transduced utilizing LVs at low MOI and short periods of exposure to the vector using a defined combination of cytokines (mSCF, FLT3-L and hTPO) and in the presence of retronectin.

In our IUT experiments, we observed higher chimerism levels, similar to those obtained with non-manipulated cells (see Supplementary Table S1), when mSCF and hIL-11 were used during transduction than when mSCF+FLT3-L+hTPO were used (Figure 4 and Supplementary Table S1). However, significant percentages of EGFP$^+$ cells were not observed by flow cytometry in recipient mice when the first combination of growth factors was used. On the other hand, the percentage of engrafted mice was lower when transplanted cells were transduced in the presence of mSCF, FLT3-L and hTPO. Under these latter conditions, we were able to detect higher proportions of EGFP$^+$ cells in the engrafted animals. These lower percentages of engrafted mice are therefore the outcome of the conditions that mediated high transduction efficiencies in the grafts (mSCF, FLT3-L, hTPO and retronectin; 6 h; MOI 10) and suggest a mechanism of cell rejection in these animals. Similar data have been recorded after the *in utero* transplantation of allogeneic BM cells[25] or transduced syngeneic adult BM.[7]

In our humoral immunoresponse assays, 61% of transplanted mice exhibited antibodies against EGFP in serum. Cell immunoresponses were also observed. Interestingly, whereas animals showing an EGFP immunoresponse did not present EGFP$^+$ cells and displayed a low exogenous engraftment capacity, mice without an anti-EGFP immunoresponse were more efficiently engrafted with transduced EGFP-expressing cells. These data indicate that engraftment failure and the loss of EGFP$^+$ cells in *in utero* transplant recipients are due, at least in part, to an immunoresponse against cells expressing the exogenous transgenic protein.

One of the proposed advantages of *in utero* cell and gene therapy is the preimmune status of the fetus, yet there are a number of indirect arguments supporting an immunologic barrier against engraftment. The fact that only immunodeficient human fetuses have obtained a clear clinical beneficial effect from IUHCT illustrates this engraftment barrier.[2,8] Moreover, T cells showing TCR rearrangement have been observed in human FL in the 13th week of gestation,[26] and FL NK cells and fetal T cells have been demonstrated alloreactive *in vitro*.[27–29] These lines of evidence could indicate the engraftment failure of FL hematopoietic cells transplanted *in utero* in allogeneic recipients.[30,31] Recent studies suggest that an immunoreaction takes place when the fetus is exposed to allogeneic cells.[10] Interestingly, this reaction has been described as caused, at least partially, by alloantibodies present in maternal milk.[32] Effectively, we detected anti-EGFP antibodies in operated mothers as early as 2 days after intrauterine transplantation, suggesting that these antibodies could be present in the maternal milk later on, thus mediating similar immunoresponses as those described for alloantibodies.[32] Moreover, if *in utero* transplanted pups are transferred to non-operated foster mothers upon delivery, no immunoresponse against EGFP is observed. Our observations therefore indicate that immunoresponses, including T-cell reactivity, against foreign transgenic proteins can be generated in an *in utero* transplantation setting and this immunoresponse has a maternal origin.

Tolerance to EGFP and other exogenous proteins has been widely described in irradiated adult mice.[33–37] Additionally, in numerous studies, *in utero* transplanted mice have revealed the long-term engraftment of transduced cells expressing transgenes.[7,11,36,38–40] Tolerance to allogeneic cells has also been described in prenatal transplants, and this has been used to induce complete chimerism after a postnatal boost.[41–43] In our experiments, a number of *in utero* transplanted mice developed tolerance to EGFP after transplantation and maintained the long-term engraftment of EGFP$^+$ cells (see G4 group in Figure 5), indicating that this phenomenon can eventually take place after *in utero* transplantation. Interestingly, animals showing EGFP$^+$ cells in the long term exhibited low proviral copy numbers per cell, suggesting that low expression of the foreign protein could facilitate immune tolerance after surgery for IUT. Thus, in the IUHCT setting, the transplant of limited numbers of transduced cells and the use of weak promoters[44] could limit the immunoresponse raised against the foreign transgene.

In summary, FL hematopoietic progenitors may prove to be a good source of cells for genetic correction and subsequent *in utero* transplantation to treat inherited diseases at very early stages. However, our findings indicate that special precaution should be taken to avoid potential maternal immunoresponses that could limit the efficacy of this therapeutic strategy.

MATERIALS AND METHODS

Mice

For adult or *in utero* hematopoietic transplants, F1 hybrids of strains B6.SJL-PtprcaPep3b/BoyJ and DBA/2J (CD45.1/CD45.2 phenotype) were used as donors, and F1 hybrids of strains C57BL/6J and DBA/2J (CD45.2/CD45.2 phenotype) as recipients. Mice were kept under high-standard conditions (HEPA-filtered air, regulated temperature of 22 °C, light/dark cycle of 12 h and allowed food and UV-irradiated water *ad libitum*) and routinely screened for pathogens. All experimental procedures were carried out according to the Spanish and European legislation (Spanish R.D 1201/2005 of the Ministry of Agricultural, Food and Fisheries concerning the protection and use of animals in scientific research; European Directive 86/606/CEE on the use and protection of vertebrate mammals used for scientific purposes).

Lentivirus supernatants

Cells (HEK-293T) were transiently transfected with three plasmids codifying the envelope and packaging viral sequences, pMD2.VSV.G, pMDLg-pRRE and pRSV-REV, and a transfer plasmid codifying the EGFP reporter gene under the

control of the cytomegalovirus promoter, pRRLsin18.PPT.CMV.EGFP.Wpre (kindly provided by Dr Naldini). Cells (293 T) were plated on 150 cm-diameter plates; transfection was conducted when confluence was about 80% as previously described.[45,46] The supernatant was collected 30 h after transfection and centrifuged to concentrate the viral particles. Virus pellets were resuspended in phosphate buffered saline (PBS) and frozen at –80 °C until use. Functional titers of infective LVs were determined in HT1080 cells, plated at 3.5×10^4 cells per well in 24-well plates and infected overnight with different dilutions of LV supernatants. Cells were washed and incubated with fresh medium, and proportions of $EGFP^+$ cells were determined 5 days later by flow cytometry, as described. Titers between 10^8 and 10^9 TU ml^{-1} were routinely obtained.

Donor FL progenitors

FL cells were obtained from 14.5-day-old fetuses. FLs were disaggregated and filtered to remove cell fragments and clusters. The cells were resuspended in PBS 1X+0.5% bovine serum albumin+2mM EDTA (PBE) and subjected to HSC enrichment by lineage depletion. Lin^- cells were sorted using the mouse Lineage Cell Depletion Kit (Miltenyi Biotech, Bergisch Gladbach, Germany) following the manufacturer's recommendations. Briefly, cells were stained with a biotinylated lineage antibody cocktail (including the lineage antigens CD5, CD45R (B220), CD11b, Gr-1 (Ly-6G/C), 7–4, and Ter-119) for 10 min at 4 °C. Next, the cells were stained again with anti-biotin MicroBeads (Miltenyi Biotech) for 15 min at 4 °C and then washed with PBE and subjected to negative immunomagnetic selection on an MS-type column (Miltenyi Biotech). Lin^- cells were washed with PBE and resuspended in PBS. On average, a 90%-pure population of Lin^- was obtained, the recovery being 30–60% of the input number of Lin^- cells.

Transduction of Lin^- cells using LVs

Lin^- cells were resuspended in Iscove's Modified Dulbecco's Medium (Biowhittacker, Walkersville, MD, USA) supplemented with 20 fetal bovine serum, 1% glutamine and 0.5% penicillin–streptomycin at a density of 5×10^5 cells/ml. Viruses were added at different MOI for different times of infection. During transduction, the medium was supplemented with one of two different combinations of cytokines: hIL-11 and mSCF (100 ng/ml each, provided by R&D Systems, Minneapolis, MN, USA), or mSCF (100 ng/ml; R&D Systems), FLT3-L (100 ng/ml; Miltenyi Biotech) and hTPO (300 ng/ml each; R&D Systems), in the absence or presence of retronectin (Takara Shuzo, Otsu, Japan). After this period of time, cells were washed to remove the viruses and resuspended in Iscove's Modified Dulbecco's Medium.

In vitro colony forming unit assays

To count myeloid colony-forming units (CFU-GM), FL cells were seeded in MethoCult GF M3534 culture medium (StemCell Technologies, Vancouver, BC, Canada), plated in triplicate on 35-mm plastic tissue culture plates (Nunc, Roskilde, Denmark) and cultured at 37 °C in 5% CO_2 and fully humidified air. After 7 days of plating, colony numbers and $EGFP^+$ colonies were scored.

Hematopoietic transplant in adult recipients

In all, 8- to 12-week-old recipients were irradiated following a myeloablation regimen (11 Gy split into two doses, 24 h apart) using a Philips MG324 X-ray device (Philips, Hamburg, Germany) set at 300 kV, 10 mA, to deliver a dose rate of 1.03. Gy/min. In all, 2 to 3×10^5 Lin^- viable-transduced cells from syngeneic 14.5-day-old FLs were transplanted into five adult recipients per group. At 30, 60 and 90 days after transplantation, peripheral blood from these animals was obtained and analyzed for chimerism by flow cytometry. For secondary transplants, 5×10^6 BM cells were injected per secondary recipient.

IUHCT

On day 14.5 after mating, pregnant females were anesthetized by inhaled isoflurane anesthesia (Sigma-Aldrich, St Louis, MO, USA). The abdomen was accessed by a midline laparotomy and the uterine horns exposed. Each fetus was injected intraperitoneally with 2 to 3×10^5 viable Lin^- cells in 5 µl of PBS using a 100 µm beveled glass micropipette. Fetuses were rehydrated with PBS and reintroduced into the peritoneal cavity, which was closed in two planes using reabsorbable suture (Ethicon PDS II 5/0; Johnson & Johnson, Brussels, Belgium). As analgesia, each mother was then injected subcutaneously with 0.15 mg kg^{-1} body weight of buprenorphine (Buprex, Reckitt Benckiser Healthcare Ltd., Nottingham, UK).

Chimerism

Chimerism was assessed in peripheral blood samples from adult and *in utero* transplanted mice (primary recipients), and from secondary recipients. Blood was taken after a small tail vein incision, and chimerism was assessed by flow cytometry, using an anti-CD45.1-PE antibody (BD Pharmigen, Palo Alto, CA, USA). A minimum number of 10^4–10^5 viable cells was acquired. Off-line analysis was performed using CXP software (Beckman Coulter, Hialeah, FL, USA). The presence of transgene-expressing cells was determined through their EGFP expression.

Detection of anti-EGFP antibodies

Mouse sera were collected and stored at –20 °C until the end of the experiment. Recombinant EGFP protein (Biovision Research Products, CA, USA) was diluted to 3 µg/ml in carbonate–bicarbonate buffer (Sigma-Aldrich) and adsorbed to enzyme-linked immunosorbent assay plates overnight (Maxisorp F96, Nunc Thermo Fisher Scientific, Roskilde, Denmark). The plates were blocked with 5% bovine serum albumin in PBS for 1 h at 4 °C. Mouse sera were diluted in PBS with 5% bovine serum albumin and added to the plates after blocking, followed by incubation for 1 h at room temperature. The plates were washed with 0.05% Tween in PBS. Bound antibodies were detected using goat anti-mouse IgM or IgG horseradish peroxidase (Sigma-Aldrich). Antibody–antigen complexes were visualized using 3,3′,5,5′-tetramethyl benzidine substrate (Pierce Biotechnology, Rockford, IL, USA). The reaction was stopped using 0.18 M H_2SO_4. Absorbance was read at 450 nm using a Tecan Genius Pro microplate reader (Tecan Trading AG, Männedorf, Switzerland). The positive signal was at least double the background absorbance. A pool of sera from wild-type mice was used as the negative control.

Analysis of EGFP-specific T-cell proliferation

Splenocytes were isolated from mouse spleen and dispersed in 10 ml of Dulbecco's modified Eagle's medium (Gibco, Grand Island, NY, USA). Erythrocytes were lysed with NH4Cl 0.84% and washed three times in Dulbecco's modified Eagle's medium (Gibco). Cell counts and viability were determined by trypan blue exclusion. A total of 2×10^5 splenocytes were incubated with 10 µg/ml recombinant EGFP at 37 °C in a humidified 5% CO2 atmosphere. On day 3, 10 U recombinant IL-2 (R&D Systems) were added. After 7 days of incubation, the cells were pulsed for the last 18 h with [3 H]^1 thymidine (1 µCi per well, Moravek Biochemicals, Brea, CA, USA). Finally, cells were harvested (Tomtec, Orange, CT, USA), and [^{3}H]^1 thymidine incorporation was measured using a β-liquid scintillation counter LKB 1205 Betaplate (Wallac, Perkin-Elmer and Analytical Sciences, Boston, MA, USA). Results are expressed as counts per minute and presented as means ± s.d. of triplicate experiments. As a proliferation control, 2×10^5 splenocytes in 200 µl of complete medium were incubated for 3 days with phytohemagglutinin (Sigma-Aldrich) at a final concentration of 10 µg/ml.

Enzyme-linked immunosorbent assay for measuring mouse interferon-γ

Interferon-γ released by activated cells was measured in supernatants collected after day 5 by cytokine enzyme-linked immunosorbent assay (Mouse interferon-γ, Bender MedSystem GmbH, Austria). The chromogenic substrate tetramethyl benzidine solution was added to the wells and the reaction stopped after 10 min with 1M phosphoric acid. Absorbance was measured at 450 nm as described above.

Analysis of EGFP DNA sequences by PCR

For conventional PCR analysis, EGFP sequences were identified using a PTC200 Peltier Thermal Cycler, MJ Research Inc. (Waltham, MA, USA). Genomic DNA was extracted using the DNAeasy Tissue Kit (QIAGEN, West Sussex, UK) according to the manufacturer's instructions. The primers for the EGFP sequence were EGFPF1: 5′-AGCTCGATGCGGTTCACCAG-3′and EGFPR1: 5′-GCCACAAGTTCAGCGTGTCC-3′. Amplification was performed

using Taq DNA polymerase and the 100 mM dNTPs set (dAT, dCTP, dGTP and dTTP) (both by Invitrogen, Life Sciences, San Diego, CA, USA). The thermal profile was one hold of 2 min at 95 °C, 40 cycles at 94 °C 30 s, 57 °C 30 s and 72 °C 15 s and a final elongation at 72 °C 1 min. PCR products were analyzed by electrophoresis in 1% agarose gel. For quantitative PCR analysis, we used the TaqMan real-time PCR procedure. EGFP copy numbers were determined by the real-time quantitative PCR approach using a Rotor Gene RG-3000 (Corbett Research Products, Foxboro, MA, USA). Primers for the EGFP sequence were as follows: QEGFPF1: 5′-GTAAACGGCCACAAGTTCAGC-3′ and QEGFPR1: 5′-TGGTGCAGATGAACTTCAGGG-3′, and detected with the TaqMan probe MGBNFQ: FAM-5′-CTTGCCGTAGGTGGC-3′-BHQ1. Amplification of the murine genomic β-actin sequence was achieved using the primers: β-actin-MF: 5′-ACGGCCAGGTCATCACTATTG-3′ and β-actin-MR: 5′-ACTATG GCCTCAAGG-AGTTTTGTCA-3′, and detected with the TaqMan probe β-actin-T: 5′-TR-AACGAGCGGTTCCGATGCCCT-BHQ2-3′. The TaqMan Universal Mastermix, NoAmpErase UNG Applied Biosystems (ROCHE, Nutley, NJ, USA) was used for amplification. The cycling conditions were one hold of 10 min at 95 °C, followed by 55 cycles of 20 s at 95 °C and 30 s at 58 °C.

Statistical analysis

Groups were compared using the Wilcoxon Mann–Whitney *U*-test. All statistical tests were performed using Statgraphics Plus 5.0 software (Manugistics Inc., Rockville, MD, USA).

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

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Fanconi anaemia: from a monogenic disease to sporadic cancer

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Abstract The dissection of the molecular pathways participating in genetic instability disorders has rendered invaluable information about the mechanisms of cancer pathogenesis and progression, and is offering a unique opportunity to establish targeted anticancer therapies. Fanconi anaemia (FA) is a paradigm of cancer-prone inherited monogenic disorders. Moreover, accumulated evidence indicates that genetic and epigenetic alterations in FA genes can also play an important role in sporadic cancer in the general population. Here, we summarise current progress in the understanding of the molecular biology of FA and review the principal mechanisms accounting for a disrupted FA pathway in sporadic cancer. Additionally, we discuss the impact of these findings in the development of new anticancer therapies, particularly with DNA interstrand cross-linkers and with new inhibitors of the FA and/or alternative DNA repair pathways.

Keywords Fanconi anaemia · Sporadic cancer · Cross-linkers · PARP inhibitors · Chronic myeloid leukemia · Acute myeloid leukemia · Synthetic lethality

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Introduction

Fanconi anaemia (FA) is an autosomal recessive disease (except for *FANCB*, which is X-linked) characterised by congenital abnormalities, progressive bone marrow failure and cancer susceptibility, mainly acute myeloid leukaemia (AML) and squamous cell carcinoma (SCC) [1]. At the cellular level, FA cells are highly sensitive to DNA inter-strand cross-linking (ICL) agents and also to oxidative stress [2].

Because of the current evidence showing the role of the FA pathway in DNA repair and cancer suppression, increasing attention is being paid to understanding the physiological consequences of mutations in FA genes with three main purposes: (1) to elucidate the role of FA genes in inherited and sporadic cancer, (2) to conduct prognostic studies of cancer progression and (3) to predict the response of cancer cells with a disrupted FA pathway to anticancer agents.

The Fanconi anaemia pathway

To date 13 different complementation groups have been characterised in FA, each of them associated with mutations in the corresponding FA gene *(FANCA, -B,- C, -D1/BRCA2, -D2, -E, -F, -G, -I, -J/BRIP1, -L, -M and N/PALB2)*. A FA-like disorder has been recently reported in patients from the same family with biallelic mutations in *RAD51C* (potentially *FANCO*) [3].

As shown in Fig. 1, the FA/BRCA pathway can be organised into three different molecular complexes in the cell nucleus: (1) the FA core complex (FACC); (2) the complex formed by FANCI and FANCD2 proteins (ID complex) and (3) proteins located downstream from the ID complex.

The FA pathway can be activated both by DNA damage and DNA replication (see review in [4]). Initially, eight FA proteins (FANCA, -B, -C, -E, -F, -G, -L and -M) together with other FA-associated proteins (FAAP24, FAAP100),

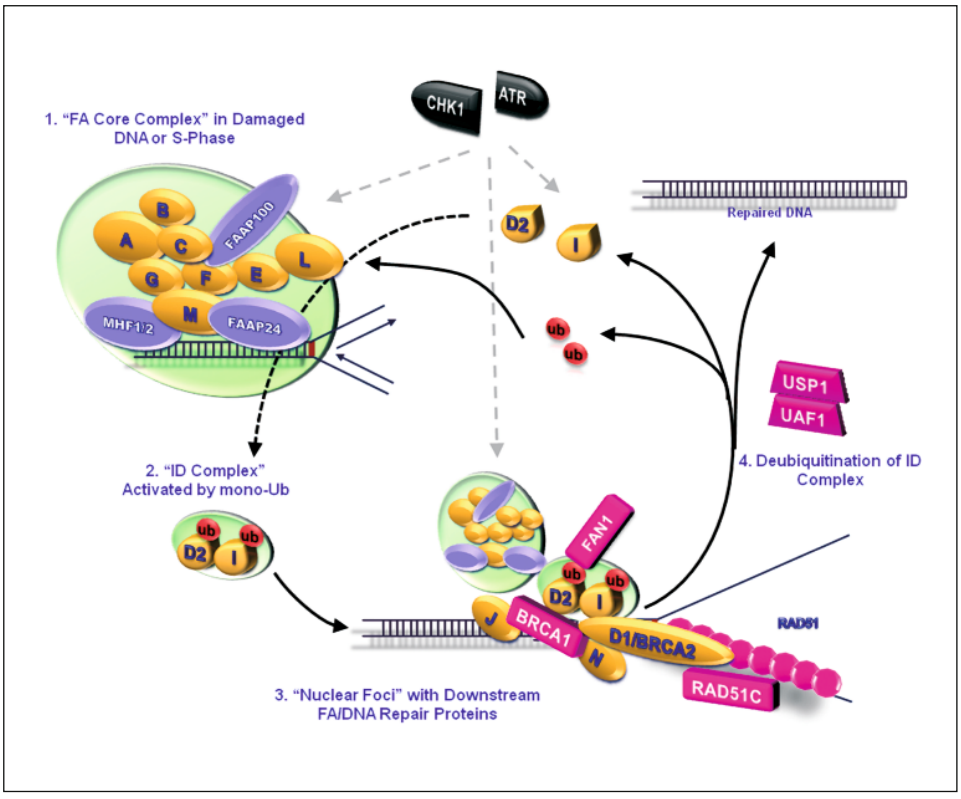

Fig. 1 Schematic representation of the FA/BRCA pathway. The generation of DNA ICL in the DNA produces a stalled replication fork. In response to the damage, ATR or its effector kinase CHK1 activates the FA/BRCA pathway. In the first step, FANCM, FAAP24 and MHF1-2 detect the ICL in the stalled fork and recruit the rest of the FA core complex. In step 2, the FA core complex, containing an ubiquitin ligase domain in FANCL, facilitates the activation of FANCD2 and FANCI via monoubiquitination (ID complex). This process promotes the loading of ID complex onto chromatin. In step 3, monoubiquitinated FANCD2 binds FAN1 and functionally interacts with downstream FA proteins and other DNA repair proteins at the damaged sites. The pathway is completed when the activated ID complex is deubiquitinated by the tandem USP1/UAF (Step 4) to resolve DNA ICLs. FA proteins are shown in yellow. Proteins also required for ubiquitination of ID complex are shown in blue. Non-FA downstream proteins participating in the FA pathway are depicted in pink. Ub, ubiquitin

MHF1 and MHF2 are assembled, forming a large nuclear ubiquitin E3 ligase complex in Fig. 1. Only two proteins of this core have a recognised catalytic activity: FANCL, with an E3 ubiquitin ligase domain [5], and FANCM, with ATP-dependent translocase activity [6]. After the generation of an ICL, the FANCM/FAAP24/MHF1-2 proteins recognise it in the stalled replication fork and recruit the FACC by direct interaction between FANCM and FANCF (see review in [4]). In a second step, the FACC facilitates the monoubiquitination of the tandem FANCD2/FANCI proteins [7, 8]. The activated ID complex is then loaded onto chromatin and binds the Fanconi-associated nuclease 1 (FAN1), which provides the nuclease activity during ICL repair in DNA-damaged sites and colocalises with downstream proteins [9–12]. These proteins include FA proteins such as FANCD1/BRCA2, FANCN/PALB2 and FANCJ/BRIP1, as well as other proteins, such as RAD51C (putative FANCO), BRCA1 and RAD51, associated with homologous recombination (HR) [13]. Also other nucleases and translesion synthesis polymerases are probably recruited by the ID complex for the processing of the ICLs (see review in [4]). Finally, the FA pathway is inactivated by the USP1/UAF1 enzyme complex, which deubiquitinates FANCD2 and FANCI, resolving the ICL damage [14].

The main upstream regulator of the FA pathway is ATR. This kinase phosphorylates directly or via its effector kinase Chk1, multiple proteins of the FA pathway and other FA-associated proteins, including FANCA, FANCE, FANCI, FANCD2 and BRCA1. These kinases coordinate the cell response to DNA damage in the S-phase. Additional molecular interactions have been reported with proteins including BLM, NBS and H2AX among others (review in [15]).

As can be deduced from the nomenclature given to proteins participating in the FA pathway, not all of them are identified as FA proteins. This is explained either because there are no FA patients whose disease can be accounted for by mutations in these genes (i.e., *FAAP24*, *FAAP100*, *MHF1*, *MHF2*), or because their mutations are associated with phenotypes that only partially overlap with the phenotype of FA patients (i.e., *ATR, BLM, NBS1*, whose mutations account for Seckel [16], Bloom [17] and Nijmegen [18] breakage syndromes, respectively).

The Fanconi anaemia pathway and sporadic cancer

The prevalence of cancer in carriers of FA mutations has been reviewed in detail by García and Benítez in this journal [19]. Defects in the FA/BRCA pathway are, however, not exclusive to inherited cancer. In this respect, different studies have already shown the relevance of FA pathway inactivation in malignancies from individuals without a family history of cancer (see review in [20]).

As shown in Table 1, the principal mechanisms that account for a disrupted FA pathway in sporadic cancer can be summarised as follows: (1) epigenetic silencing of FA genes, (2) somatic mutations leading to loss of function of FA

Table 1 Sporadic malignancies with a disrupted FA pathway

Aberration in tumour	Gene	Cancer type	Frequency (%)	Reference
Promoter hypermethylation	*BRCA2*	Breast	9/18 (50)	[65]
	BRCA2	*Granulosa*	1/25 (4)	[25]
	FANCB	HNSCC	1/16 (6.2)	[66]
	FANCC	AML	1/143 (0.7)	[67]
	FANCC	ALL	3/97 (3.1)	[67]
	FANCF	*AML*	1	[21]
	FANCF	Bladder	1/41 (2.4)	[27]
	FANCF	*Bladder*	1/23 (4.3)	[27]
	FANCF	Breast	13/75 (17.3)	[68]
	FANCF	Breast	1/120 (0.8)	[26]
	FANCF	Cervical	27/91 (29.7)	[23]
	FANCF	*Cervical*	3/9 (33.3)	[23]
	FANCF	Germ cell (non-seminoma)	4/60 (6.7)	[69]
	FANCF	*Granulosa*	6/25 (24)	[25]
	FANCF	HNSCC	13/89(14.6)	[24]
	FANCF	*NSCLC*	22/158 (13.9)	[24]
	FANCF	Ovarian	4/19 (21)	[70]
	FANCF	*Ovarian*	5/18 (27.7)	[22]
	FANCF	Ovarian[a]	7/53 (13.2)	[70]
	FANCF	*Ovarian*	1/7 (14.3)	[71]
	FANCF	*Ovarian*	2/25 (8)	[22]
	FANCF	*Ovarian*	1/9 (11.1)	[70]
	FANCL	ALL	1/97 (1.0)	[67]
	FANCN	Breast	4/60 (6.6)	[72]
	FANCN	Ovarian	4/53 (7.5)	[72]
Somatic mutation	*BRCA2*	Breast	1/69 (1.4)	[73]
	BRCA2	Breast	1/23 (4.3)	[74]
	BRCA2	Ovarian[b]	4/92 (4.3)	[37]
	FANCA	AML	4/101 (3.4)	[31]
	FANCA	AML	5/79 (6.3)	[32]
	FANCC	Hepatocellular[c]	1/5 (20)	[35]
	FANCC	Pancreatic[a]	2/33 (6.1)	[34]
	FANCD2	Oral SCC	7/21 (33.3)	[75]
	FANCG	Oral SCC	6/21 (28.6)	[75]
	FANCG	Pancreatic[a]	1/33 (3.0)	[34]
Absent mRNA without hyphermethylation detected	*BRCA2*	Ovarian	1/18 (5.5)	[76]
	BRCA2	Ovarian[b]	12/92 (13.0)	[37]
Splicing variants	*FANVL*	Lung	13/25 (52)	[41]
	FANVL	Prostate	30/45 (66.6)	[41]
	FANVL	Osteosarcoma	10/10 (100)	[41]

Data extracted and modified from [77] and from N. Ameziane PhD thesis 2009. Studies marked in italics: only cell lines were used.

[a]LOH in *FANCC* or *FANCG* were detected in 11 unselected pancreatic cell lines and a selection of 22 tumours from young-onset pancreatic cancer

[b]*BRCA2* mRNA was not detectable in 12 cases of ovarian sporadic cancer, one of which showed *BRCA2* promoter methylation. Familial cancer cases are also included in this cohort.

[c]No healthy tissue samples from the patient are available to determine the origin of the homozygous mutation.

genes and (3) elevated expression of splicing variants which downregulate and compete with functional FA proteins.

The FA genes that have been found inhibited in cancer cells by promoter hypermethylation include *FANCF, FANCL, FANCC, FANCB, BRCA2/FANCD1* and *FANCN/PALB2* (see Table 1). Different studies have shown that hypermethylation of *FANCF* is the most frequent epigenetic modification that leads to FA pathway disruption in different cancer types, including AML [21], ovarian cancer [22], cervical cancer [23], head and neck squamous cell carcinoma (HNSCC), non-small cell lung cancer (NSCLC) [24], granulosa cell tumours [25], breast cancer [26] and bladder carcinoma [27]. Other studies, however, did not observe FA gene hypermethylation in ovarian tumours [28], HNSCC [29] and AML [30], indicating significant discrepancies in the frequency of this process in sporadic cancer.

Somatic mutations in FA genes have been observed in different malignancies. In two independent studies, deletions [31] or point mutations [32] were found in *FANCA* in sporadic AML. Dysfunctions in *FANCA* have also been

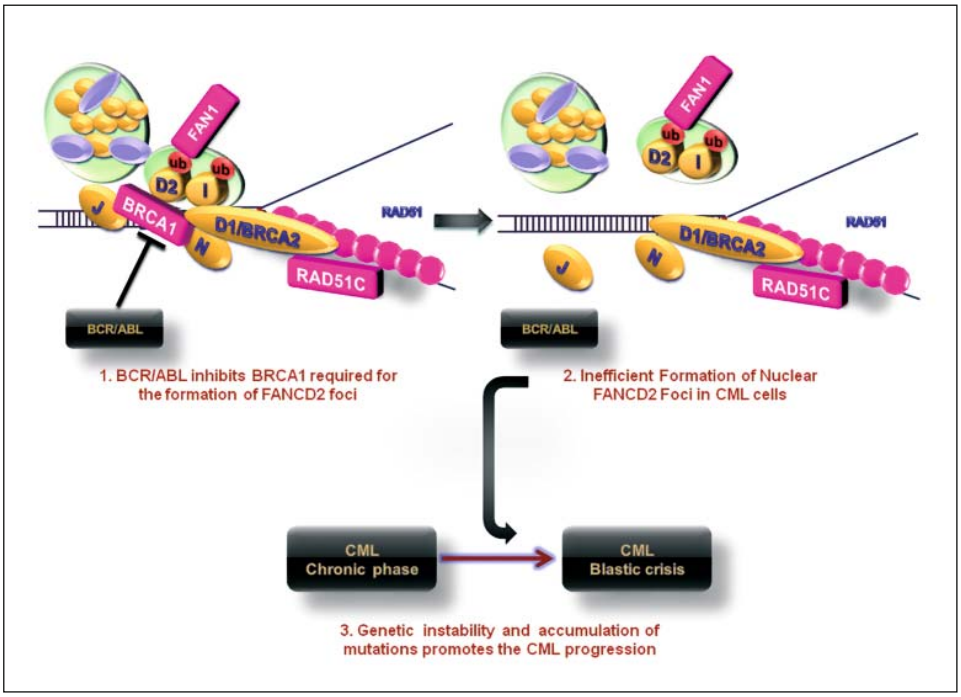

Fig. 2 Proposed model of FA/BRCA disruption by BCR-ABL. (1) BCR-ABL downregulates BRCA1 expression. (2) Inhibited formation of FANCD2 foci in response to a downregulated expression of BRCA1 after DNA damage. (3) As a consequence of the defective FA pathway, the genetic stability of the cell is compromised, inducing accumulation of mutations that promote the transition from a chronic phase to blast stage in a CML precursor cell

described in another AML, although the pathogenic mutations were not identified in this study [33]. Also rare somatic mutations in *FANCC* and *FANCG* have been observed in pancreatic cancer [34]. Recently, a homozygous inactivating mutation in *FANCC* has been identified in a hepatocellular carcinoma cell line (HCC), although the authors did not find genetic inactivation of *FANCC* in subsequent analyses of 18 HCC specimens [35]. In the case of ovarian tumours, a similar prevalence of somatic mutations of *BRCA2* was found in two different studies (4% [36] and 4.3% [37], respectively). In breast cancer patients, loss of heterozygosity (LOH) together with acquired somatic mutations in *BRCA* genes have also been reported [38–40].

A new mechanism leading to FA pathway disruption relies on the elevated expression of a splicing variant of *FANCL*, termed *FAVL*. Strikingly, in their study, Zhang et al. observed that *FAVL* was expressed in 50% of tested carcinoma cell lines and primary carcinomas, and that expression of FAVL mediated a decrease in FANCL expression. Moreover, FAVL provided cancer cells with a growth advantage, caused chromosomal instability and promoted tumour development in a xenograft mouse model [41].

The relevance of the Fanconi anaemia pathway in chronic myeloid leukaemia

Chronic myeloid leukaemia (CML) constitutes one of the best models to investigate the progression from an early stage of cancer development (chronic phase) towards an advanced stage (blast crisis), characterised by a marked chromosomal instability. To gain insights into the mechanisms involved in the genetic instability of CML, our group has recently investigated the functionality of the FA pathway in haematopoietic progenitors (CD34+ cells) from patients with CML and also in healthy CD34+ cells from cord blood transduced with the *BCR/ABL* oncogene (the causative agent of CML). Either of these cell types showed a defective capacity to generate FANCD2 nuclear foci after DNA damage. Additionally, we observed that the BCR/ABL oncogene generated centrosomal amplification and induced chromosomal instability in CD34+ cells. Because the monoubiquitination of this protein was unaffected in *BCR/ABL* cells, the FA pathway defect mediated by the *BCR/ABL* oncogene was placed downstream from the ID complex. Moreover, the impaired formation of FANCD2 nuclear foci and also the chromosomal instability induced by *BCR/ABL* were both reverted by the ectopic expression of *BRCA1*–a protein which is markedly downregulated in *BCR/ABL*-expressing cells [42, 43]. A model showing the relationship between the FA pathway and the genetic instability mediated by *BCR/ABL* is proposed in Fig. 2. In this model, *BCR/ABL* downregulates the expression of BRCA1, interfering with the efficient translocation of FANCD2 to chromatin and therefore its colocalisation with other proteins conferring genetic stability to the cell. As a consequence of such genetic instability, it is proposed that CML cells progressively accumulate mutations, thus contributing to the progression from a chronic phase towards blast crisis [44].

Significance of the Fanconi anaemia pathway in the cell response to anticancer drugs

Drugs producing DNA interstrand cross-links (ICL drugs) block the progression of the replication fork and interfere

with both the replication and the transcription processes of the cell [45–47]. Examples of these drugs are bifunctional alkylating agents like mitomycin C (MMC), diepoxybutane (DEB) or cisplatin [48].

In 2003, Taniguchi et al. [22] described for the first time the influence of the methylation status of a FA promoter, the *FANCF* promoter, in the response of an ovarian cancer cell line to cisplatin, and proposed a model where the methylation of *FANCF* mediated the chromosomal instability of the cells. While initially *FANCF*-deficient cancer cells were hypersensitive to cisplatin, the continuous exposure of the cells to the drug resulted in the generation of clones where *FANCF* was demethylated, and therefore resistant to cisplatin.

As discussed before, defects of the FA/BRCA pathway in cancer cells have also been related to the expression of FA variant proteins, such as the *FAVL* variant [41]. As is the case in conventional FA cells, cell lines expressing high levels of *FAVL* were hypersensitive to MMC, an observation with evident implications in the development of optimised cancer therapies with DNA ICL agents. Very recently, Villarroel et al. have shown that the inactivation of *PALB2* was determinant of the response to DNA damage in pancreatic cancer, identifying an additional biomarker FA gene to predict the response of pancreatic cancer cells to DNA cross-linkers, MMC and cisplatin [49].

In our laboratory, we observed that FA-deficient cells were hypersensitive to trabectedin [50], a new marine anticancer drug indicated for soft tissue sarcoma and ovarian cancer [51]. Trabectedin binds to the DNA minor groove by establishing a covalent bond in one strand of the DNA and one or two hydrogen bonds in the opposite strand [52], mimicking the interaction produced by classical ICL drugs. Our observations suggested the relevance of investigating the response of cancer cells to new ICL-like drugs and of conducting individualised studies on the functionality of the FA pathway in tumours to predict their response to classical ICLs and new ICL-like drugs.

Based on high sensitivity of cells with non-functional *FA/BRCA* genes to ICL drugs, recent studies aimed to interfere with the FA/BRCA pathway using specific inhibitors. This is the case of a natural anti-cancer compound, curcumin, to inhibit FANCD2 monoubiquitination, or the synthetic agent wortmannin, which inhibits kinases, such as ATR and ATM, upstream in the FA/BRCA pathway [53]. Aiming to improve the potency and specificity of these inhibitors, new compounds, such as monoketone, an analogue of curcumin [54], or the naphthoquinone (DDN) [55], have been more recently developed. Similarly, other approaches aim to sensitise head and neck human cancer cell lines to cisplatin by reducing BRCA1 expression with the histone deacetylase inhibitor phenylbutyrate [56]. Other studies using high-throughput screening showed new agents as candidates to target cancer cells with a deficient FA pathway [57].

With the purpose of improving the selectivity of anticancer drugs without harming healthy cells, the concept of "synthetic lethality" has recently gained importance in anticancer therapy. Two genes are synthetic lethal if mutation of either alone is compatible with viability, but mutation of both genes leads to death [58]. This concept has been proposed as a new alternative for inducing selective anticancer therapy by targeting different DNA-damage repair pathways. This would be particularly useful for the treatment of patients where only the cancer cells are deficient in a particular DNA-repair pathway. By means of the pharmacological blocking of a different DNA-repair pathway, it would be possible to induce the specific killing of the tumour. This is the case of the Chk1 inhibitor Gö6976, which is synthetically lethal with FA-deficient tumours [59]. Other examples are the poly(ADP-ribose) polymerase (PARP) inhibitors, which showed selective cytotoxicity in BRCA2-deficient cells [60, 61]. Significantly, cells deficient in FANCA, FANCC or FANCD2 also show hypersensitivity to this compound [62]. Based on this type of observation, PARP inhibitors have been used in clinical trials, either alone or in combination with DNA cross-linkers [63].

Concluding remarks

Since the first descriptions showing the role of FA genes in DNA repair, the involvement of a disrupted FA pathway in inherited and sporadic cancer has been progressively shown. Further studies should be conducted aiming to define more precisely the frequency of the different processes leading to FA pathway disruption in different sporadic malignancies. Understanding whether a disruption in the FA pathway constitutes an early event in cancer development or whether it rather mediates secondary events conferring genetic instability–as has been respectively shown in ovarian cancer cells [22] and CML [44]–should be also clarified in different cancer cell types to better understand the role of FA pathway disruption in cancer. Additionally, the extent to which a disruption in the FA pathway would imply a hypersensitive response of the tumour to ICL agents needs careful evaluation, due to the multitude of mechanisms that a cancer cell may develop to induce chemoresistance. A good example is the observation that BCR/ABL both interferes with the FA/BRCA pathway and also with apoptotic pathways, thus conferring chemoresistance to ICLs [44]. Another interesting example showing ICL resistance in cells with a disrupted FA pathway relies on the acquired abrogation of the G2 checkpoint. As proposed by Ceccaldi et al., this mechanism would allow cells with biallelic mutations in FA genes to survive despite spontaneous DNA damage, although this could be produced at the expense of an increased risk of cell transformation [64]. Finally, in the coming years we will have the opportunity to evaluate the clinical impact of new inhibitors of the FA pathway and also of drugs mediating synthetic lethality in different malignancies.

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Conflict of interest Sandra Martínez is a PharmaMar employee and the other authors declare no conflict of interest relating to the publication of this manuscript.

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Research Article

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Targeted gene therapy and cell reprogramming in Fanconi anemia

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Abstract

Gene targeting is progressively becoming a realistic therapeutic alternative in clinics. It is unknown, however, whether this technology will be suitable for the treatment of DNA repair deficiency syndromes such as Fanconi anemia (FA), with defects in homology-directed DNA repair. In this study, we used zinc finger nucleases and integrase-defective lentiviral vectors to demonstrate for the first time that *FANCA* can be efficiently and specifically targeted into the *AAVS1* safe harbor locus in fibroblasts from FA-A patients. Strikingly, up to 40% of FA fibroblasts showed gene targeting 42 days after gene editing. Given the low number of hematopoietic precursors in the bone marrow of FA patients, gene-edited FA fibroblasts were then reprogrammed and re-differentiated toward the hematopoietic lineage. Analyses of gene-edited FA-iPSCs confirmed the specific integration of *FANCA* in the *AAVS1* locus in all tested clones. Moreover, the hematopoietic differentiation of these iPSCs efficiently generated disease-free hematopoietic progenitors. Taken together, our results demonstrate for the first time the feasibility of correcting the phenotype of a DNA repair deficiency syndrome using gene-targeting and cell reprogramming strategies.

Keywords cell reprogramming; Fanconi anemia; gene-targeting; iPSCs; zinc finger nucleases

Subject Categories Genetics, Gene Therapy & Genetic Disease; Haematology; Stem Cells

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Introduction

The progressive development of engineered nucleases has markedly improved the efficacy and specificity of targeted gene therapy, opening new possibilities for the treatment of inherited and acquired diseases in the clinics (Tebas *et al*, 2014). In contrast to conventional gene therapy with integrative vectors, targeted gene therapy enables the insertion of foreign sequences (i.e., therapeutic genes or small oligonucleotides) in specific sites of the cell genome. Thus, depending on the genetic etiology of the disease, the gene-targeting approach may pursue the correction of a specific mutation or, alternatively, the insertion of the therapeutic transgene into safe loci of the genome, often referred to as 'safe harbors' (Naldini, 2011).

In spite of the advances in the field, the question of whether or not targeted gene therapy will be applicable to diseases where homology-directed repair (HDR) is affected has never been explored. Taking into account that Fanconi anemia (FA) proteins participate in HDR (Taniguchi *et al*, 2002; Yamamoto *et al*, 2003; Niedzwiedz *et al*, 2004; Yang *et al*, 2005; Nakanishi *et al*, 2011) and coordinate the action of multiple DNA repair processes, including the action of different nucleases and homologous recombination (see reviews in Kee & D'Andrea, 2010; Kottemann & Smogorzewska, 2013; Moldovan & D'Andrea, 2009), we aimed to investigate for the first time the possibility of conducting a targeted gene therapy strategy in FA cells.

Genetically, FA is a complex disease where mutations in sixteen different genes (*FANC-A*, *-B*, *-C*, *-D1/BRCA2*, *-D2*, *-E*, *-F*, *-G*, *-I*, *-J/BRIP1*, *-L –M*, *–N/PALB2*, *-O/RAD51C; -P/SLX4; -Q/ERCC4/XPF*) have been reported (Bogliolo *et al*, 2013). Among all these genes, mutations in *FANCA* account for about 60% of total FA patients (Casado *et al*, 2007; Auerbach, 2009). Importantly, while few recurrent mutations (i.e., truncation of exon 4 in Spanish gypsies or mutations

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in exons 13, 36, and 38) have been observed in FA-A patients, *FANCA* mutations are generally private mutations, which include point mutations, microinsertions, microdeletions, splicing mutations and large intragenic deletions (Castella *et al*, 2011). Thus, considering the large number of genes and mutations that can account for the FA disease, the insertion of a functional FA gene in a 'safe harbor' locus would lead to the generation of a targeted gene addition platform with a broad application in FA, regardless of the complementation group and mutation type of each patient.

Recent studies by our group and others aiming at the identification of 'safe harbor sites' in the human genome have shown robust and stable expression of transgenes integrated in the human *PPP1R12C* gene, a locus also known as *AAVS1*, across different cell types (Smith *et al*, 2008; Lombardo *et al*, 2011). Additionally, no detectable transcriptional perturbations of the *PPP1R12C* and its flanking genes were observed after integration of transgenes in this locus, indicating that *AAVS1* may represent a safe landing path for therapeutic transgene insertion in the human genome (Lombardo *et al*, 2011). These observations, together with the development of artificial zinc finger nucleases (ZFNs) that efficiently and selectively target the *AAVS1* locus, have facilitated gene editing strategies aiming at inserting therapeutic transgenes in this locus, not only in immortalized cell lines but also in several primary human cell types, including induced pluripotent stem cells (hiPSCs; Hockemeyer *et al*, 2009; DeKelver *et al*, 2010; Lombardo *et al*, 2011; Zou *et al*, 2011b; Chang & Bouhassira, 2012).

Because a defective FA pathway not only predisposes FA patients to cancer (Rosenberg *et al*, 2008) but also to the early development of bone marrow failure due to the progressive extinction of the HSCs (Larghero *et al*, 2002; Jacome *et al*, 2006), our final aim in these studies was the generation of gene-edited, disease-free FA-HSCs, obtained from non-hematopoietic tissues of the patient. Thus, in our current studies, we firstly pursued the specific insertion of the therapeutic *FANCA* gene in the *AAVS1* locus of FA-A patients' fibroblasts. Thereafter, gene-edited FA cells were reprogrammed to generate self-renewing disease-free iPSCs and finally re-differentiated toward the hematopoietic lineage, as previously described with FA cells corrected by conventional LV-mediated gene therapy (Raya *et al*, 2009).

Our goal of conducting a combined approach of gene editing and cell reprogramming in FA cells was particularly challenging taking into account the relevance of the FA pathway both in HDR (Taniguchi *et al*, 2002; Yamamoto *et al*, 2003; Niedzwiedz *et al*, 2004; Yang *et al*, 2005; Moldovan & D'Andrea, 2009; Kee & D'Andrea, 2010; Nakanishi *et al*, 2011; Kottemann & Smogorzewska, 2013) and cell reprogramming (Raya *et al*, 2009; Muller *et al*, 2012; Yung *et al*, 2013). In spite of these hurdles, the strong selective growth advantage characteristic of corrected FA cells allowed us to establish a new approach for the efficient generation of FA HPCs harboring specific integrations of the therapeutic *FANCA* gene in a safe harbor locus.

Results

Efficient gene-targeting-mediated complementation of fibroblasts from FA-A patients

To promote insertion of a *FANCA* expression cassette into the *AAVS1* locus, an integrase-defective lentiviral vector (IDLV) harboring the *EGFP* and *FANCA* transgenes flanked by *AAVS1* homology arms (donor IDLV) was generated (Fig 1A top). In this donor IDLV, *FANCA* is under the transcriptional control of the human PGK promoter. In addition, a promoterless *EGFP* cDNA preceded by a splice acceptor (SA) site and a translational self-cleaving 2A sequence was also included upstream of the *FANCA* cassette. Upon targeted-mediated insertion into *AAVS1*, the *EGFP* cassette will be placed under the transcriptional control of the promoter of the ubiquitously expressed *PPP1R12C* gene, thus allowing the FACSorting of gene-targeted cells (Fig 1A). Besides the donor IDLV, an adenoviral vector expressing a ZFN pair (AdV5/35-ZFN), designed to induce a DNA double-strand break in the *AAVS1* locus, was used to enhance the efficiency of gene targeting in this locus (Hockemeyer *et al*, 2009).

To investigate the feasibility of performing gene targeting in FA-A cells, skin fibroblasts from four FA-A patients with different mutations in *FANCA* were transduced either with the donor IDLV alone, or with the donor IDLV and the AdV5/35-ZFNs simultaneously. Fourteen days after transduction, cells were analyzed by flow cytometry to measure the proportion of $EGFP^+$ fibroblasts. While <0.05% of the cells transduced with the donor IDLV alone were positive for EGFP, 0.2–1.1% of FA fibroblasts that had been co-transduced with the donor IDLV and the ZFNs-AdV were $EGFP^+$ (See Fig 1B and representative analyses in Supplementary Fig S1). Strikingly, the percentage of $EGFP^+$ cells markedly increased during the *in vitro* culture of these cells, reaching levels between 5.5 and 13.4% (Fig 1B), showing the proliferation advantage of gene-edited FA-A fibroblasts.

Because the prolonged *in vitro* culture of FA fibroblasts results in increased rates of cell senescence (Muller *et al*, 2012), in a new set of experiments, fibroblasts from three FA patients (FA-52, FA-123 and FA-644) were transduced with an excisable h*TERT*-expressing LV (Salmon *et al*, 2000) prior to performing the gene-targeting procedure. Transduction of FA fibroblasts with hTERT-LVs resulted in a marked increase in telomerase activity (see representative data in Supplementary Fig S2). Significantly, the proportion of $EGFP^+$ cells was markedly increased (3–4-fold) in *hTERT*-transduced versus untransduced FA fibroblasts from FA patients (Fig 1C), indicating that hTERT improved the efficacy of gene targeting in FA-A fibroblasts. Consistent with data obtained with non-immortalized fibroblasts, when immortalized gene-edited FA fibroblasts were maintained in culture, a progressive increase in the proportion of $EGFP^+$ cells was also observed (see data from geFA-52T in Fig 1D). Strikingly, around 40% of treated FA-A fibroblasts were $EGFP^+$ after 42 days in culture in the absence of any selectable drug (Fig 1D).

PCR analyses with two pairs of primers that amplify, respectively, the 5′ and the 3′ integration junctions between the *EGFP/FANCA* cassette and the endogenous *AAVS1* locus evidenced the insertion of the *EGFP/FANCA* cassette into the *AAVS1* locus of sorted $EGFP^+$ geFA-52T fibroblasts (Fig 1E). In these gene-edited FA fibroblasts, the activity of hTERT was also confirmed (Supplementary Fig S2).

To investigate whether the insertion of the therapeutic h*FANCA* cassette in the *AAVS1* locus of FA-A fibroblasts corrected the cellular phenotype of the disease, the functionality of the FA pathway in FA-52T fibroblasts was tested both before (negative control) and after the gene-targeting procedure. As a positive control, healthy

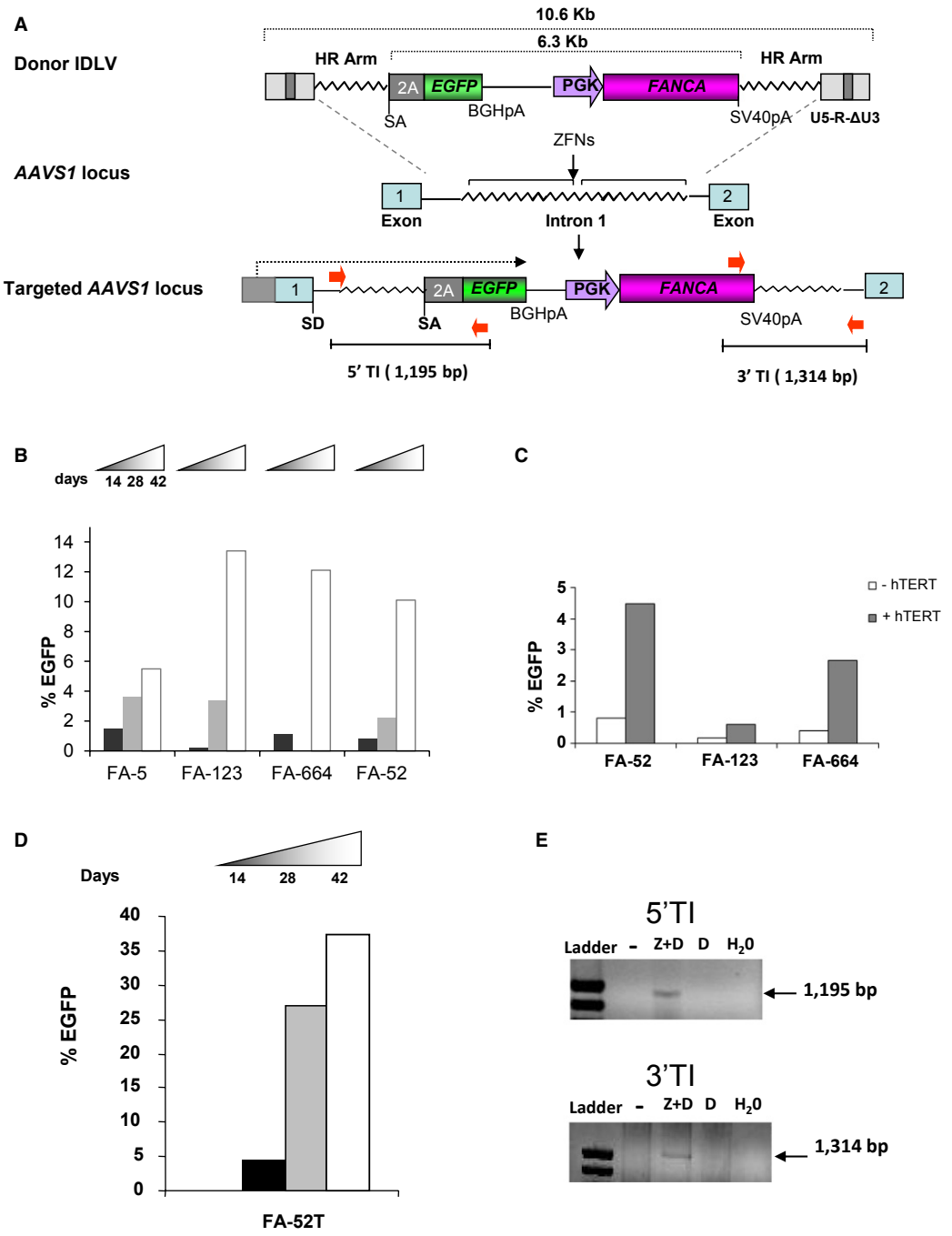

Figure 1. Efficacy of gene targeting of *FANCA* in the *AAVS1* locus of primary hFA-A fibroblasts.

A Top: schematic representation of the donor integrase-defective lentiviral vector (IDLV) used to promote insertion of the *EGFP/FANCA* cassette into the *AAVS1* locus. Middle: *AAVS1* locus with the zinc finger nucleases (ZFNs) target site. Bottom: *AAVS1* locus upon ZFN-mediated targeted insertion of the *EGFP/PGK-FANCA* cassette. Black arrow shows transcription of the *EGFP* from the endogenous *PPP1R12C* promoter. HA, homology arm; SD, splice donor; SA, splice acceptor; BGHpA, bovine growth hormone polyadenylation signal; SV40pA, simian virus 40 polyadenylation signal. Constituents of the LTR (U5-R-ΔU3) are also indicated.

B Proliferation advantage of targeted Fanconi anemia (FA) fibroblasts ($EGFP^+$ cells) during *in vitro* incubation.

C Comparative analysis of gene targeting in FA-A fibroblasts, untransduced or transduced with a lentiviral vector expressing h*TERT*. Analyses were performed 14 days after gene targeting.

D *In vitro* proliferation advantage of targeted FA fibroblasts ($EGFP^+$) previously transduced with hTERT (FA-52T fibroblasts).

E Targeted integration analysis of the *EGFP/PGK-FANCA* cassette into the *AAVS1* site by PCR using primers specific for the 5′ or 3′ integration junctions (red arrows in the top schematic) defined as 5′ TI or 3′ TI, respectively.

donor fibroblasts (H.D. Fib) were analyzed in parallel. The presence of nuclear FANCD2 foci, fully dependent on the expression of all the FA core complex proteins, including FANCA (Garcia-Higuera *et al*, 2001), was determined in these samples after DNA damage induced by mitomycin C (MMC). In contrast to uncorrected FA-52T fibroblasts (FA-52T Fib.), which did not generate FANCD2 foci even after MMC exposure, a significant proportion of the geFA-52T fibroblasts generated FANCD2 foci, mainly after treatment with MMC, thus mimicking the response of H.D. fibroblasts (Fig 2A). Because the main characteristic of FA cells is the increased chromosomal instability upon exposure to DNA inter-strand cross-linking (ICL) drugs, we also investigated the response of both uncorrected and gene-edited FA-A fibroblasts to diepoxybutane (DEB). While in FA-52T fibroblasts DEB induced a significant increase in the number of chromosomal aberrations per cell (from 0.05 ± 0.05 to 1.7 ± 0.46 aberrations/cell)— including chromatid breaks and

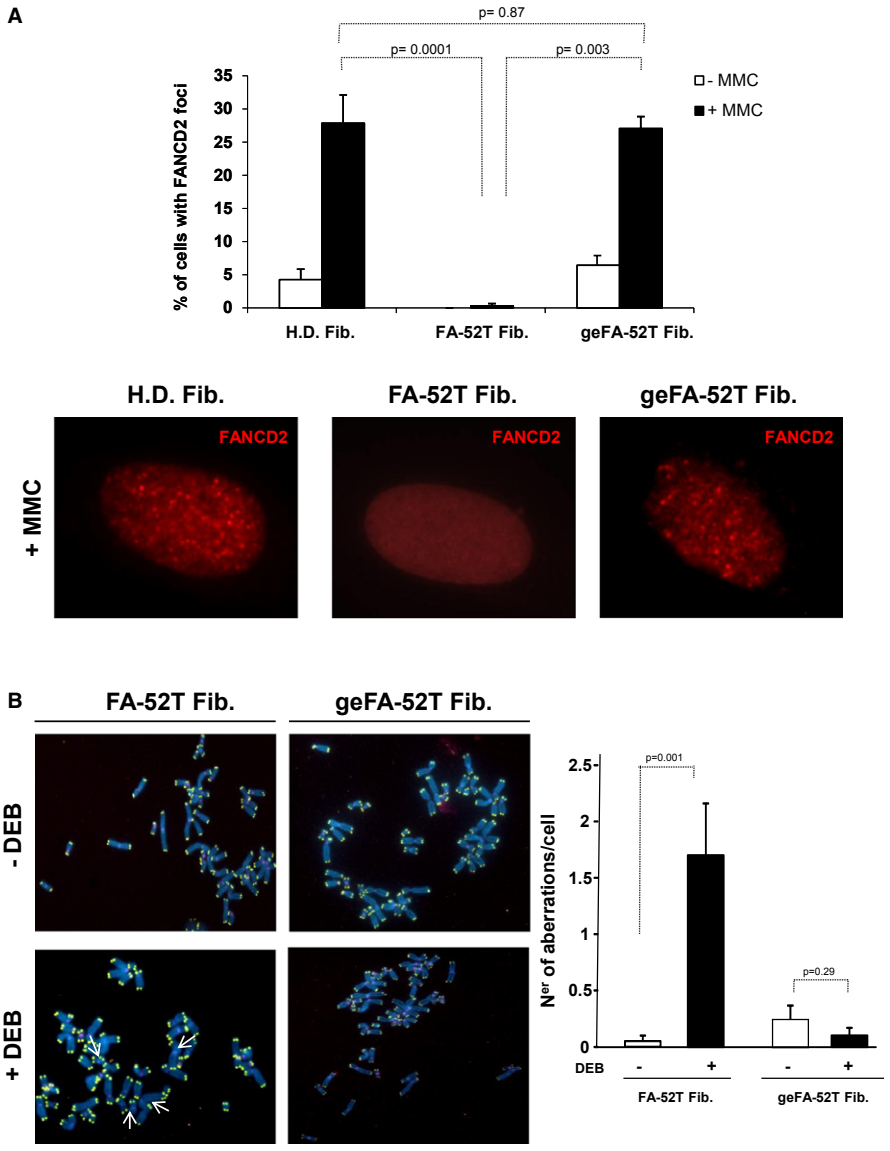

Figure 2. Phenotypic correction of the gene-edited FA-A fibroblasts.

A Top: histogram showing the percentage of FA-A fibroblasts, unstransduced or co-transduced with the donor integrase-defective lentiviral vector (IDLV) and the AdV5/35-ZFNs (geFA-52T Fib), showing FANCD2 foci in the absence or the presence of mitomycin C (MMC). Bottom: representative images of FANCD2 foci (red) in cells shown in the top histogram, after MMC treatment.

B Chromosomal instability induced by diepoxybutane (DEB) in untreated (FA-52T) and gene-edited FA fibroblasts (geFA-52T Fib). Left: representative FISH analysis was performed by staining telomeres (in green), centromeres (in pink) and chromosomes (in blue). Right: histogram showing the number of chromosomal aberrations per cell.

Data information: Values are shown as mean ± s.e. from three independent experiments (A) or analysis of twenty different metaphases per group (B). All *P*-values were calculated using two-tailed unpaired Student's *t*-test.

radial chromosomes, typically found in FA patients' cells—the same DEB treatment did not induce any increase in the number of chromosomal aberrations in geFA-52T fibroblasts (Fig 2B).

Taken together, these results show the feasibility of correcting the phenotype of FA cells using gene targeting strategies, in particular by promoting the insertion and expression of *FANCA* in the *AAVS1* safe harbor locus of fibroblasts from FA-A patients.

Efficient generation of disease-free iPSCs from FA fibroblasts corrected by gene targeting

To generate disease-free FA-iPSCs, FA fibroblasts subjected to gene editing (geFA-123, geFA-52 and geFA-52T) were first enriched for $EGFP^+$ cells by cell sorting and then reprogrammed using a polycistronic excisable LV expressing the human *SOX2, OCT4, KLF4,* and *cMYC* transgenes from the *EF1A* promoter (STEMCCA vector; Somers *et al*, 2010). Consistent with previous observations (Raya *et al*, 2009), uncorrected FA fibroblasts did not generate iPSCs after reprogramming, even after transduction with the TERT-LV (data not shown). Although several iPSC-like colonies were generated from gene-edited FA-123 fibroblasts (115 AP^+ cells/100,000 fibroblasts), no stable iPSC lines could be generated from FA fibroblasts simply subjected to gene editing, most probably because of the pro-senescence nature of these cells. In marked contrast to these observations, the reprogramming of FA fibroblasts that were first transduced with the hTERT-LV and then subjected to gene editing generated 230 iPSC-like clones, most of which could be maintained after serial *in vitro* passages (Supplementary Fig S3). Twelve iPSC clones generated from geFA-52T fibroblasts were further expanded and differentiated into fibroblasts to perform additional studies to confirm the integration site of the EGFP/*FANCA* construct. First, qPCR analyses were conducted to determine the mean copy number per cell of the *EGFP/FANCA* cassette. As shown in Supplementary Table S1, 11 out of the 12 geFA-iPSC clones analyzed were positive for *EGFP* integration and contained an average of 0.98 ± 0.44 *EGFP* copies per cell. The only iPSC clone that did not harbor any EGFP copy (clone 5) did not progress more than six passages in culture.

To investigate whether the *EGFP/FANCA* cassette was specifically integrated in the *AAVS1* locus of all these iPSC clones, 3′ primers previously used in analyses of Fig 1E were used. As shown in Supplementary Table S1, all iPSC clones that were positive for integration of the cassette were also positive for the PCR band corresponding to the specific insertion in the *AAVS1* locus.

Three geFA-iPSC clones (clones 16, 26 and 31) were selected for further characterization. The pluripotency of these gene-corrected clones was first analyzed both by alkaline phosphatase (AP) staining and immunohistochemistry staining of different pluripotency genes. Representative pictures in Fig 3A and Supplementary Fig S4A showed that all tested geFA-iPSCs clones were highly positive for AP, NANOG, TRA-1-60, OCT4, and SSEA-4 expression. RT-qPCR analyses of the expression of endogenous pluripotency genes *NANOG, OCT4, SOX2, KLF4,* and *cMYC* were consistent with the pluripotent nature of these clones (Supplementary Fig S4B). In all cases, a very low expression of the ectopic reprogramming transgenes was found, indicating substantial inactivation of the *EF1A* promoter present in the reprogramming vector. As expected for *bona fide* iPSC clones, *OCT4* and *NANOG* promoters were hypomethylated in gene-corrected FA-iPSC clones, in clear contrast to the high level of methylation observed in H.D. fibroblasts (Supplementary Fig S4C). To further demonstrate the pluripotency of geFA-iPSC16 cells *in vivo*, cells were subcutaneously inoculated in NSG mice. Characteristic teratomas containing complex structures representing the three embryonic germ layers were observed 8–10 weeks after implantation. Immunofluorescence staining confirmed the expression of definitive endoderm markers (Fox2A), neural structures that expressed neuroectodermal markers (ß-III-tubulin) and the generation of mesoderm (Brachyury) and mesoderm derivatives tissue such as muscle (α-SMA; Fig 3B).

To confirm the insertion of the FANCA cassette into the *AAVS1* locus in the gene-corrected FA-iPSC clones, Southern blot analyses were performed on genomic DNA extracted from gene-edited geFA-iPSC clones 16, 26, and 31. Blots hybridized with probes for the exogenous *EGFP* and the endogenous *AAVS1* genes confirmed the monoallelic integration of the *EGFP/FANCA* cassette into the *AAVS1* locus and the absence of random integration in any of the three tested clones (Fig 3C,D).

Once demonstrated the generation of *bona fide* gene-edited FA-iPSCs, in the next set of experiments, we aimed to verify whether these geFA-iPSCs were disease free, as shown for their parental gene-edited FA fibroblasts (Fig 2). First, we verified by qRT-PCR that h*FANCA* mRNA levels corresponding to the three tested geFA-iPSC clones were very similar to levels observed in the control ES cell line and markedly higher when compared to uncorrected FA-52T fibroblasts (Fig 4A). Western blot analysis confirmed the expression of FANCA in all the three tested clones (Fig 4B). Even more, since FANCA is necessary for the relocation of FANCD2 to damaged DNA sites, we investigated the presence of nuclear FANCD2 foci in three geFA-iPSC clones exposed to MMC. As shown in Fig 4C, these analyses further confirmed the expression and functionality of FANCA in the three tested geFA-iPSC clones. Consistent with the restored FA pathway of gene-edited FA-iPSCs, DEB did not induce a significant increase in the number of chromosomal aberrations in FA-corrected cells. Remarkably, the number of chromosomal aberrations in geFA-iPSCs (0.2 ± 0.1 aberrations/cell; Fig 4D) was ten times lower to the number observed in their parental uncorrected fibroblasts (see Fig 2B).

To assure the identity of the different geFA-iPSC clones, the presence of the original pathogenic mutations described in patient FA-52 (c.710-5T>C and c.3558insG) was investigated by Sanger sequencing both on FA-52T fibroblasts and geFA-iPSC clones 16, 26, and 31 (Supplementary Fig S5). The confirmation of both pathogenic mutations in the three tested geFA-iPSCs, together with our observations showing that all stable iPSC clones contained the *AAVS1*-targeted *FANCA* gene (Supplementary Table S1) and had a functional FA pathway, demonstrates that the disease-free nature of gene-edited FA-iPSCs is a consequence of the functional insertion of *FANCA* within the *AAVS1* safe harbor site of these reprogrammed FA cells.

Aiming to excise the STEMCCA vector from the genome of geFA-iPSCs, cells from clone 16 were transduced with an IDLV co-expressing the Cre recombinase and the Cherry fluorescence marker (Papapetrou *et al*, 2011). Thereafter, individual colonies were isolated to select those clones with a lower number of copies of the STEMCCA provirus. Two clones were selected: Excised clones

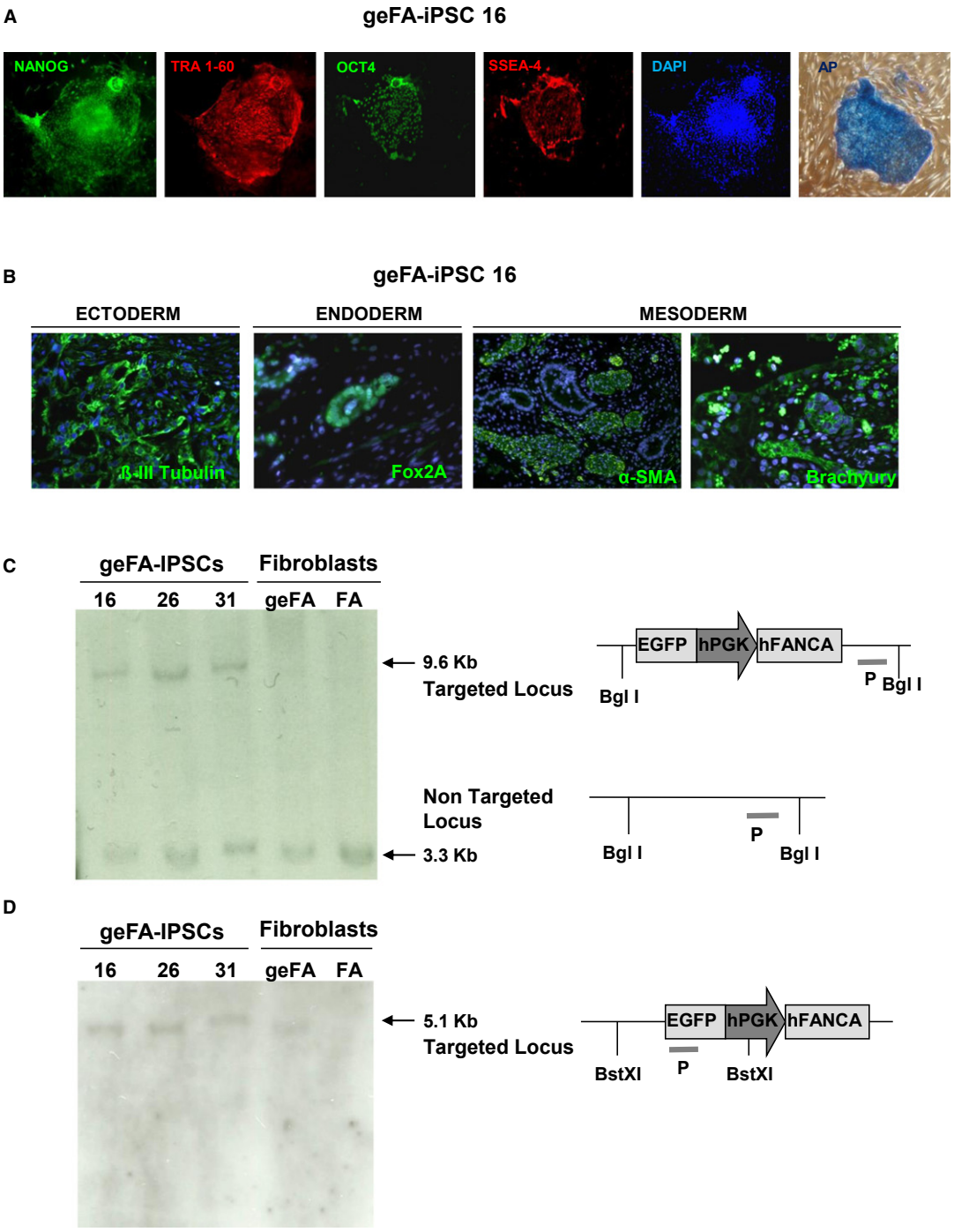

Figure 3. Pluripotency characterization and insertion site analyses of gene-edited FA-A iPSCs.

A Expression of TRA1-60, SSEA-4, OCT4, and NANOG pluripotency markers by immunofluorescence staining of gene-edited FA-iPSCs (geFA-iPSCs; clone 16).

B Immunofluorescence analysis of ectoderm (β-II-tubulin), endoderm (Fox2A), and mesoderm (α-SMA and Brachyury) in teratomas generated from geFA-iPSCs (clone 16).

C Southern blot analysis of genomic DNA extracted from the indicated gene-corrected FA iPSC clones (geFA-IPSCs) and from parental fibroblasts, either unmanipulated (FA) or after gene editing (ge-FA iPSCs, clones 16, 26 and 31). Genomic DNA was digested with BglI and hybridized with a probe for *PPP1R12C*. The band of 9.6 kb corresponds to the targeted integration in *PPP1R12C*, while the 3.3 kb correspond to the untargeted allele.

D Southern blot analysis of samples shown in (C) digested with BstXI and hybridized with a probe (P) for *EGFP*. One single band of 5.1 kb is expected for specific integrations in *PPP1R12C*.

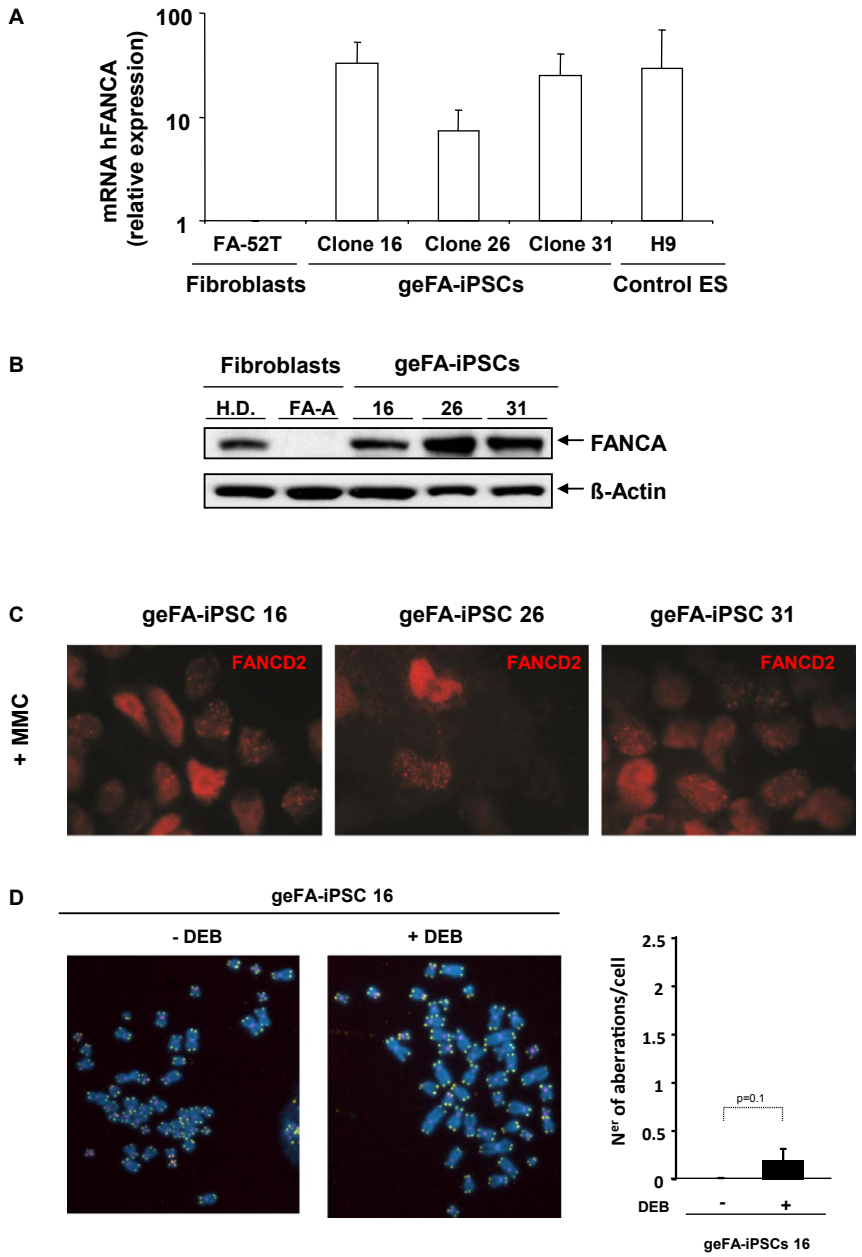

Figure 4. Disease-free Fanconi anemia phenotype of corrected geFA-iPSCs.

A Histogram showing the levels of *hFANCA* expression in gene-edited FA-iPSC clones and human ES (H9) relative to untreated FA-52T fibroblasts. Data are shown as mean ± s.e. of three different analyses.

B Western blot analysis showing FANCA expression in geFA-iPSC clones in comparison with fibroblasts from HD and a FA-A patient.

C Representative immunofluorescence analysis of FANCD2 foci in geFA-iPSCs after DNA damage with mitomycin C (MMC).

D Chromosomal instability induced by diepoxybutane (DEB) was also tested in geFA-iPSC 16. FISH analysis was performed using probes to detect telomeres (green), centromeres (pink) and chromosomes (blue). Right: histogram showing the number of chromosomal aberrations per cell.

Data information: Data are shown as mean ± s.e. from three different experiments (A) or analysis of twenty different metaphases per group (D). All *P*-values were calculated using two-tailed unpaired Student's *t*-test.

16.1 and 16.2, with a number of 0.35 ± 0.10 and <0.05 copies/cell, respectively. In clone 16.2, the excision of the h*TERT* provirus was also confirmed (<0.05 copies as deduced from q-PCR analyses). RT-qPCR analysis performed in these two subclones showed the persistent expression of endogenous pluripotency genes (*SOX2, OCT4, KLF4, NANOG, and cMYC*) and the absence of ectopic transgenes expression (Supplementary Fig S6A). As expected from *bona fide* pluripotent iPSC clones, these two clones generated teratomas with structures characteristics of the three germ layers (Supplementary Fig S6B).

Analysis of the genetic stability of gene-edited FA fibroblasts and iPSCs

Because of the chromosomal instability of FA cells, we investigated by means of karyotype analyses and aCGH analyses whether the different manipulations of FA-52 fibroblasts and their corresponding iPSCs induced chromosomal instability. As shown in Table 1, no evident karyotype or aCGH abnormalities were observed in expanded FA-52 parental fibroblasts when compared with a reference human DNA sample. Even more, the transduction with *hTERT-LV* and the gene-editing process did not induce evident chromosomal abnormalities in these cells. Reprogrammed geFA-52 iPSCs also had a normal karyotype, although a deletion in the 16p12.2p12.1 locus was noted in the aCGH analysis. After excision with the Cre recombinase, in addition to the 16p deletion, a mosaic trisomy in chromosome 5 was observed (See Table 1 and Supplementary Fig S7).

Generation of disease-free hematopoietic progenitors from gene-edited FA-A iPSCs

In experiments corresponding to Fig 5 and Supplementary Figs S8 and S9, we investigated whether hematopoietic progenitor cells derived from gene-edited FA-iPSCs were disease-free. To conduct these experiments, embryoid bodies from geFA-iPSCs were incubated with hematopoietic cytokines as described in Materials and methods. As shown in representative analyses from Supplementary Fig S8A, the hematopoietic differentiation of geFA-iPSCs after 21 days of *in vitro* stimulation was demonstrated by the presence of hematopoietic precursors ($CD43^+/CD34^+$), committed hematopoietic progenitors ($CD34^+/CD45^+$) and also mature hematopoietic cells ($CD34^-/CD45^+$). When the hematopoietic differentiation of excised and non-excised iPSC clones was compared, the proportion of $CD45^+$ and $CD34^+/CD45^+$ was consistently increased in the case of the excised *vs* the non-excised clones (see data from two independent experiments in Fig 5A and Supplementary Fig S8). Consistent with the flow cytometry data, granulo-macrophage and erythroid colonies were generated by geFA-iPSC-differentiated cells in methylcellulose. As it was observed in the flow cytometry studies, higher numbers of hematopoietic progenitors were generated by excised versus non-excised geFA-iPSC (Fig 5B). In all instances, colonies derived from geFA-iPSC were almost as resistant to MMC as healthy cord blood progenitor cells, in contrast to the MMC hypersensitivity observed in BM progenitors from FA patients (Fig 5C).

Finally, to investigate whether gene-edited FA-iPSCs were also able to differentiate toward the hematopoietic lineage *in vivo*, one of the teratomas generated by the excised geFA-52 iPSCs (clone 16.2) was analyzed for the presence of human hematopoietic markers. As shown in Supplementary Fig S9, 3% of the cells present in this teratoma consisted on $hCD45^+/mCD45^-$ cells. Within this population, 3.5% corresponded to $hCD34^+$ cells, thus revealing the *in vivo* differentiation potential of this clone.

Table 1. aCGH analysis in FA-52 fibroblasts prior to and after gene editing and in gene-edited IPSCs-derived clones

Cells	aCGH result			Karyotype
	Alteration	Locus	OMIM GENES	
FA-52 fibroblasts[a]	–	–	–	46 XY
geFA-52T fibr.[b]	–	–	–	46 XY
geFA-52T iPSC clones				
Clone 16[c]	Deletion	16p12.2p12.1	*	46 XY
Clone 16 Ex[c]	Deletion	16p12.2p12.1	*	46 XY
	Mosaic trisomy	5	–	46 XY

*EEF2K, CDR2, HS3ST2, SCNN1G, SCNN1B, COG7, GGA2, EARS2, NDUFAB1, PALB2, DCTN5, PLK1, ERN2, PRKCB, CACNG3, RBBP6.
[a]Comparison analyses between expanded fibroblasts from patient FA-52 (FA-52 fibroblasts) and a reference male DNA sample.
[b]Comparison analyses between expanded, TERT-transduced, and gene-edited FA-52 fibroblasts (geFA-52T fibr.) with respect to FA-52 fibroblasts.
[c]Comparison analyses between geFA-52T iPSCs clone 16 and clone 16 Ex (after excision of the reprogramming cassette) and FA-52 fibroblasts.

Discussion

Thanks to the development of artificial nucleases capable of generating DNA double-strand breaks (DSBs) in pre-determined sequences of the genome (Porteus & Baltimore, 2003; Urnov *et al*, 2010; Cong *et al*, 2013; Joung & Sander, 2013), targeted gene therapy is entering into the clinics (Tebas *et al*, 2014). Whether these approaches will be amenable to the treatment of DNA repair deficiency syndromes such as FA is, however, uncertain. In this respect, it is currently known that FA proteins participate in maintaining the genomic stability of the cell and coordinate the actions of multiple repair processes, including HDR (Kottemann & Smogorzewska, 2013), making these cells particularly appropriate for investigating the feasibility of performing targeted gene therapy in syndromes associated with DNA repair defects and genome instability. Although the mechanisms explaining how the FA pathway promotes HDR are still unclear, most evidence suggests that the monoubiquitination of FANCD2—which is critically dependent on the presence of all the FA core complex proteins, including FANCA—is essential for the recruitment of several HDR factors (such as BRCA1, BRCA2, and RAD51) to damaged chromatin (see review in Kee & D'Andrea, 2010).

To investigate whether gene targeting was feasible in FA cells we focused on the most frequent FA complementation group, FA-A (Casado *et al*, 2007; Auerbach, 2009), and investigated the possibility of inserting the therapeutic transgene in a safe harbor locus of the human genome—the *AAVS1* locus (Lombardo *et al*, 2011).

Strikingly, our first results in Fig 1 clearly demonstrate the feasibility of performing gene targeting in FA-A cells with significant efficacies (up to 4%), comparable with efficacies reported in primary cells competent for DNA repair (DeKelver *et al*, 2010; Lombardo *et al*, 2011; Sebastiano *et al*, 2011; Soldner *et al*, 2011; Zou *et al*, 2011a). The feasibility of performing gene targeting in FA-A cells could be explained by different hypotheses. First, as previously described in other systems (Matrai *et al*, 2011; Peluffo *et al*, 2013), a transient though early expression of FANCA may be induced by the donor IDLV, thus facilitating the insertion of the exogenous therapeutic cassette through a HDR process. Besides

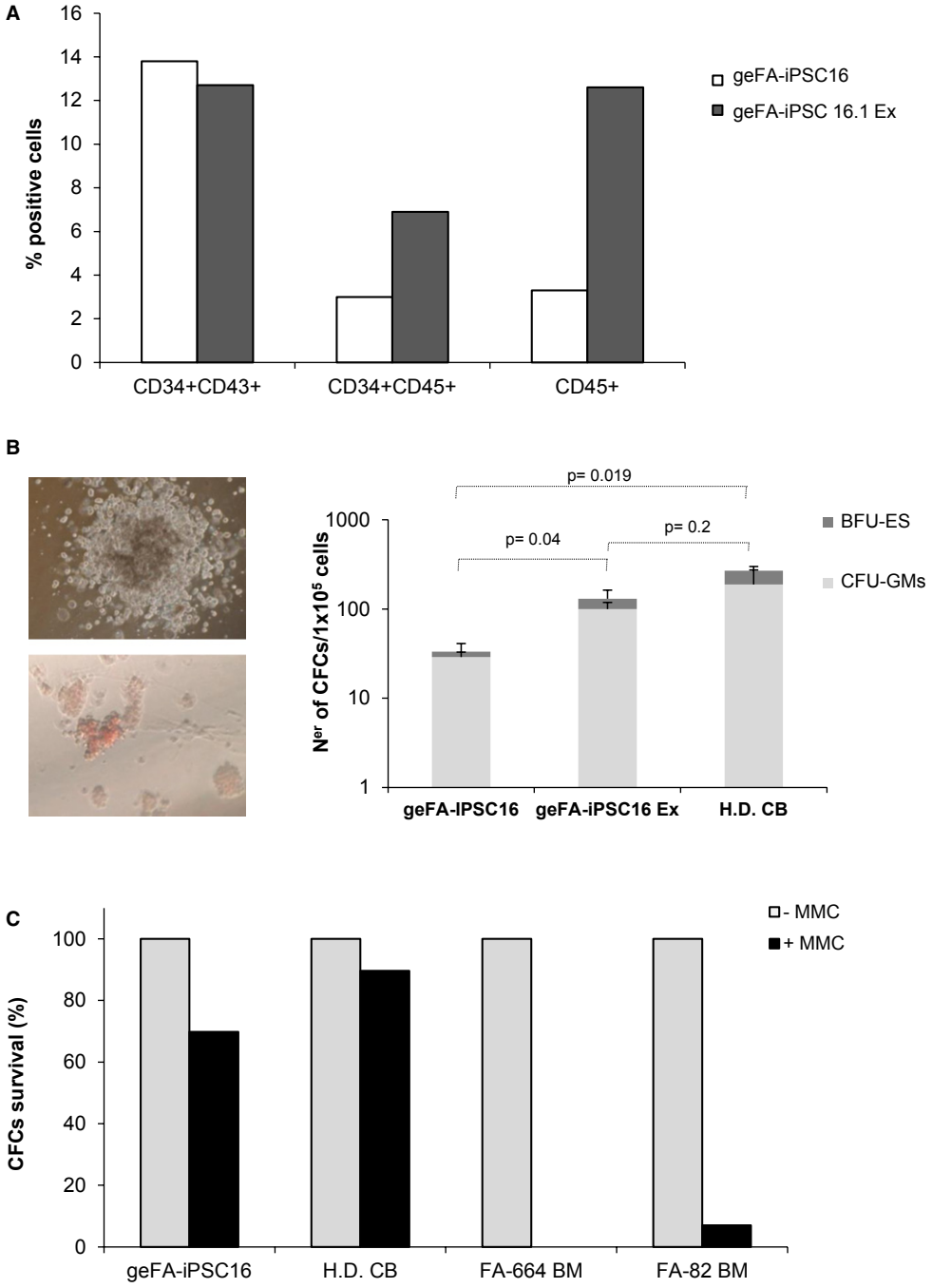

Figure 5. Hematopoietic differentiation of gene-edited FA-IPSCs.

A Analysis of the percentage of $CD43^+CD34^+$, $CD45^+CD34^+$, and $CD45^+$ cells generated by unexcised and excised geFA-iPSCs (clones 16 and Ex 16.1).

B Left: Representative pictures of hematopoietic colonies generated by geFA-iPSCs. Right: Analysis of the clonogenic potential of unexcised and excised ge-FAiPSCs (clones 16 and Ex 16.1) in comparison with H.D. cord blood cells.

C Survival to mitomycin C (MMC) of CFCs obtained from geFA-IPSCs (clone 16) in comparison with BM CFCs from two different FA patients (FA-664 BM and FA-82 BM) and with CFCs from a healthy cord blood (H.D. CB).

Data information: Values are shown as mean ± s.e. of three experiments. All *P*-values were calculated using two-tailed unpaired Student's *t*-test.

this hypothesis, we should contemplate the possibility that the limited HDR activity of FA-A cells (Nakanishi *et al*, 2005, 2011) could be sufficient to facilitate the ZFN-mediated integration of our donor IDLV in the *AAVS1* site. Finally, although the integration of the therapeutic cassette in the *AAVS1* locus might have occurred through an HDR-independent process, as reported in other models (Anguela *et al*, 2014), PCR and Southern blot analyses showed the expected amplicons and band length for targeted

integration of the cassette, strongly suggesting that *AAVS1* targeting took place through a HDR mechanism. In this respect, while the specificity of gene targeting might be reduced in FA cells, our data clearly show that all the FA-iPSC clones harbored one single copy of *FANCA* specifically integrated in the *PPPR12C* target gene (Table 1). Consequently, this result further supports the efficacy and the specificity of our gene targeting approach.

With the main objective of preventing the predisposition to senescence of FA cells (Muller *et al*, 2012), the transduction of hTERT-LV in FA-A fibroblasts induced an unexpected effect in these cells, which consisted of a significant increase in the efficacy of gene editing (Fig 1). Whether or not this effect is specific for FA cells or whether it is simply mediated by the enhanced proliferation rate of TERT-transduced FA cells is currently unknown. Nevertheless, to the best of our knowledge, the improved gene targeting mediated by hTERT observed in our experiments constitutes a new finding that has not been previously reported in any other experimental model. The observation that transduction with hTERT also facilitates the generation of gene-edited FA-iPSCs is consistent with previous data showing the relevance of hTERT in cell reprogramming (Batista *et al*, 2011; Pomp *et al*, 2011; Winkler *et al*, 2013). In safety terms, even though the *hTERT* provirus could be efficiently excised from transduced cells with the Cre recombinase, further approaches based on the transient expression of hTERT during gene editing and/or cell reprogramming would constitute safer approaches to limit potential genomic insults during the *ex vivo* manipulation of the samples.

Interestingly, EGFP analyses in gene-edited FA fibroblasts showed that in the absence of any artificial selection process, a progressive increase in the proportion of targeted cells (up to 40% after 42 days in culture) was observed, mimicking the improved growth proliferation properties of FA precursor cells in mosaic patients (Waisfisz *et al*, 1999; Gregory *et al*, 2001; Gross *et al*, 2002) or in experimental models of FA gene therapy (Rio *et al*, 2008). Consistent with previous observations in FA cells corrected by LV-mediated gene therapy (Raya *et al*, 2009), this proliferation competence of FA-corrected cells was particularly remarkable when samples were subjected to cell reprogramming, confirming the relevance of the FA pathway during the process of iPSC generation. Similar conclusions were obtained in two additional studies (Muller *et al*, 2012; Yung *et al*, 2013), although these studies showed that reprogramming of FA cells can occur, albeit with a very low efficiency compared to gene-complemented FA cells.

Studies in Figs 2 and 4 showing the generation of nuclear FANCD2 foci and the chromosomal stability of gene-edited FA fibroblasts and iPSCs upon exposure to ICL drugs demonstrate that the specific targeting of *FANCA* in the *AAVS1* locus has completely corrected the phenotype of FA-A fibroblasts and *bona fide* iPSCs. Although transduction of FA fibroblasts with the hTERT-LV might have had consequences upon the genetic instability of FA cells, our karyotype and aCGH studies indicate that neither the expansion nor the transduction with hTERT-LV or the gene-editing processes induced evident chromosomal abnormalities in FA fibroblasts. In contrast to these results, data in Table 1 and Supplementary Fig S7 showed the presence of chromosomal abnormalities in reprogrammed and excised geFA-iPSCs. Importantly, different genetic defects have also been reported in non-FA-iPSCs (Mayshar *et al*, 2010; Gore *et al*, 2011; Laurent *et al*, 2011; Cheng *et al*, 2012; Ruiz *et al*, 2013) that were associated with the generation of the iPSCs (Mayshar *et al*, 2010; Gore *et al*, 2011; Hussein *et al*, 2011; Laurent *et al*, 2011) and/or with mutations that pre-existed in the somatic population of origin (Young *et al*, 2012). This indicates that the presence of chromosomal abnormalities in our iPSCs is not exclusive of their FA genetic background and that the different mechanisms accounting for mutations in non-FA-iPSCs would be applicable to our geFA-iPSCs.

Consistent with the previous study showing the generation of disease-free FA-iPSCs through conventional gene therapy approaches (Raya *et al*, 2009; Muller *et al*, 2012), our new study shows the efficient hematopoietic differentiation of gene-edited FA-iPSCs. Moreover in the current study, we observed the generation of increased numbers of hematopoietic progenitors from geFA-iPSCs subjected to excision of the reprogramming cassette, confirming previous observations showing that the residual expression of reprogramming genes limits the iPSC differentiation potential (Ramos-Mejia *et al*, 2012). The hematopoietic differentiation observed in these experiments and the robust expression of *FANCA* targeted into the safe harbor *AAVS1* locus should account for the generation of a high number of hematopoietic progenitors with normalized response to MMC.

In summary, our study demonstrates for the first time the possibility of conducting efficient and precise targeted-mediated gene therapy in HDR-deficient cells. Moreover, we show the feasibility of reprogramming these cells to generate iPSC-derived gene-edited hematopoietic progenitors characterized by a disease-free phenotype. Our approach thus constitutes a new proof-of-concept with a potential future clinical impact to optimize the generation of gene-corrected HSCs from non-hematopoietic tissues of patients with inherited diseases, including DNA repair deficiency and genetic instability syndromes, like FA.

Materials and Methods

Cell lines and primary fibroblasts from FA-A patients

293T and HT1080 cells (ATCC: CRL-11268 and ATCC: CCL-121) were used for the production and titration of the LVs, respectively. Cells were grown in Dulbecco's modified medium GlutaMAX™ (DMEM; Gibco) supplemented with 10% fetal bovine serum (FBS, Biowhitaker) and 0.5% penicillin/streptomycin solution (Gibco). Skin fibroblasts were obtained from FA-5, FA-123, FA-664, and FA-52 patients and were maintained in DMEM (Invitrogen) supplemented with 20% FBS (Biowhitaker) and 1% penicillin/streptomycin solution (Gibco) at 37°C under hypoxic conditions (5% of O_2) and 5% of CO_2. Patients were classified as FA-A patients as previously described (Casado *et al*, 2007). The ES4 and H9 (NIH Human Embryonic Stem Cell Registry, http://stemcells.nih.gov/research/registry/) lines of hES cells were maintained as originally described (Raya *et al*, 2008). FA patients and healthy donors were encoded to protect their confidentiality, and informed consents were obtained in all cases according to Institutional regulations of the CIEMAT. All studies conformed the principles set out in the World Medical Association Declaration of Helsinki.

Vectors

pCCL.sin.cPPT.AAVS1.loxP.SA.2A.GFP.pA.loxP.PGK.FANCA.pA.Wpre donor transfer LV (donor IDLV) was generated using elements from the backbones pCCL.PGK.FANCA.Wpre* (Gonzalez-Murillo *et al*, 2010) and pCCLsin.cPPT.AAVS1.2A.GFP.pA (Lombardo *et al*, 2011). The integrase-defective third-generation packaging plasmid pMD.Lg/pRRE.D64Vint was used to produce IDLV particles (Lombardo *et al*, 2007). pLM.CMV.Cherry.2A.Cre (Papapetrou *et al*, 2011) and pLox.TERT.ires.TK vectors (Salmon *et al*, 2000) were provided by Addgene. For reprogramming experiments, the EF1α STEMCCA lentiviral vector kindly provided by Dr Mostoslavsky was used (Sommer *et al*, 2010). This vector contains the cDNAs for *OCT4*, *SOX2*, *c-MYC,* and *KLF4* flanked by loxP sequences for their subsequent excision. ZFNs targeting intron 1 of the *PPP1R12C* gene were expressed from an Adenoviral Vector (AdV5/35) under the control of the CMV promoter (Lombardo *et al*, 2011).

Cell transduction

For gene editing experiments, fibroblasts from FA-A patients were transduced either with donor IDLV alone (150 ng HIV Gag p24/ml) or together with AdV5/35-ZFNs (multiplicity of infection (MOI) 200). Fourteen days post-transduction, the proportion of $EGFP^+$ cells was determined by flow cytometry (BD LSRFortessa cell analyzer, Becton Dickinson Pharmingen). To immortalize fibroblasts from FA-52 to FA-123 patients, 10^5 cells were transduced at MOI 1 with the pLox.TERT.ires.TK LV (Salmon *et al*, 2000) for 24 h. To excise the reprogramming cassette and hTERT from established hiPSCs, single cell suspensions were generated by incubation with accutase (Gibco) and transduced for 10 h with the IDLV pLM.CMV.Cherry.2A.Cre. Immediately after transduction, 2×10^4 cells/10 cm^2 dish, expressing Cherry protein, were sorted and new subclones of the parental geFA-IPSCs were generated.

Hematopoietic differentiation

iPSC colonies were detached using colagenase type IV (Gibco) for 30 min at 37°C, washed and centrifuged at 200× *g*, resuspended in differentiation media composed by KO-DMEM (Gibco) supplemented with 20% non-heat-inactivated FBS (Biowhitaker), 1% NEAA (Lonza; Biowhitaker), L-Glu (1 mM; Invitrogen), β-mercaptoethanol (0.1 mM; Gibco) and hrBMP4 (0.5 ng/ml; Prepotech) and plated in ultra-low attachment plates (Costar). After 2 days, media were replaced by Stempro 34 (Invitrogen) supplemented with 0.5% pen/streptomicin, L-Glu (2 mM; Invitrogen), MTG (40 mM; Sigma), ascorbic acid (50 μg/ml; Invitrogen), hrSCF, hrFlt3 ligand and TPO (100 ng/ml; EuroBioSciences), hrIL3 (10 ng/ml; Biosource), hrIL6 (10 ng/ml; Prepotech), hrBMP4 (50 ng/ml; Prepotech), Wnt11 (200 ng/ml; R&D), and rhVEGF (5 ng/ml; Prepotech). Media were changed every 3–4 days. At day 7, media were replaced by fresh media where rhWnt-11 was substituted by rhWnt-3a (200 ng/ml; R&D). Media were changed every 3–4 days. At day 14 and 21, immunophenotypic analysis of the differentiated cells was performed by flow cytometry, and colony-forming unit assays were conducted (See Supplementary Methods).

Flow cytometry

Transduction with the AdV5/35-ZFNs and the donor IDLV, was analyzed by flow cytometry analysis (FACSCalibur; Becton Dickinson Pharmingen). Immunophenotypic analysis of the hematopoietic differentiated cells was performed using the following antibodies according to the manufacturer's instructions: phycoerythrin (PE)-Cy7-conjugated anti-human CD34 (BD Pharmingen), PE-conjugated anti-human CD31 (eBiosciences), allophycocyanin (APC)-conjugated anti-human CD45 (BD), and fluorescein isothiocyanate (FITC)-conjugated anti-human CD43 (BD). Fluorochrome-matched isotypes were used as controls. 4′,6-Diamidino-2-phenylindole (DAPI; Roche)-positive cells were excluded from the analysis. Analysis was performed using FlowJo software.

Inmunofluorescence and Western blot of Fanconi anemia proteins

Analyses of FANCD2 foci were performed by immunofluorescence of primary fibroblasts or iPSCs treated for 16 h with 200 nM of MMC. After MMC treatment, cells were stained with rabbit polyclonal anti-FANCD2 (Abcam, ab2187-50) as previously described (Hotta & Ellis, 2008; Raya *et al*, 2009). Cells with more than ten foci were scored as positive. FANCA expression was analyzed by Western blot (Raya *et al*, 2009) using the following antibodies: hFANCA (ab5063 Abcam) and anti-beta Actin to mouse antibody (ab6276, Abcam) as control. Goat polyclonal antibody to rabbit IgG (HRP; ab6721-1; Abcam) and sheep polyclonal antibody to mouse IgG—H&L (HRP; ab 6808, Abcam) were used as secondary antibodies. Protein quantification was done with Image J software.

FANCA expression by qRT-PCR

The expression of human *FANCA* mRNA was analyzed in the different clones of geFA-iPSCs by real-time quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR; Gonzalez-Murillo *et al*, 2010) using primers described in Supplementary Methods. Parental fibroblasts from FA-52 and ES H9 were used as controls.

Gene targeting analysis: PCR and Southern blots

For PCR analysis, genomic DNA was extracted with DNeasy Blood & Tissue Kit (Qiagen). To detect the targeted integration of the HDR cassette in the *AAVS1* locus, two different pair of primers for the 3′ or the 5′ integration junction (5′ TI and 3′ TI, respectively) were used (Supplementary Table S2). PCR was conducted as follows: 2 min at 94°C, 40 cycles of 30 s at 94°C, 30 s at 58°C (5′ TI) and 59°C (3′ TI), 1 min at 72°C and one final step for 5 min at 72°C. The proper target integration amplified a 1195 pb amplicon for the 5′ TI and a 1314 pb fragment for the 3′ TI that were resolved in agarose gel at 2%. For Southern blot analyses, genomic DNA was extracted and digested either with *Bst*XI enzyme or with *Bgl*I (both from New England Biolabs). Matched DNA amounts were separated on 0.8% agarose gel, transferred to a nylon membrane (Hybond XL, GE Healthcare) and probed either with the ^{32}P-radiolabeled sequence of a fragment of EGFP to detect specific (5.1 kb) and non-specific integrations or with a probe of *AAVS1* gene located outside of the

The paper explained

Problem
Gene targeting is becoming a true alternative to conventional gene therapy with integrative gammaretroviral or lentiviral vectors. It is however unknown whether these approaches would be applicable to inherited syndromes like FA, characterized by homology-directed DNA repair (HDR) defects. Additionally, the existence of 16 different FA genes, each of them with multiple mutations potentially accounting for the disease, would imply the necessity of developing individualized targeted gene therapy strategies in FA patients.

Results
We have demonstrated for the first time an efficient and specific targeting of *FANCA* in the *AAVS1* safe harbor locus of FA-A patients' fibroblasts. This approach allowed us to develop a gene-editing platform applicable to all FA subtypes and FA gene mutations based on the insertion of the therapeutic FA gene in a *safe harbor* locus. Moreover, gene-edited FA-A fibroblasts were reprogrammed to generate disease-free iPSCs, which could be re-differentiated toward the hematopoietic lineage in a process that resulted in the generation of gene-edited, disease-free, hematopoietic progenitor cells.

Impact
Our data showing that gene targeting is feasible in FA opens the possibility of using similar strategies in different inherited syndromes characterized by defects in HDR and genome instability. The generation of disease-free HSCs through the specific insertion of therapeutic transgenes in a safe harbor locus of non-hematopoietic cell tissues, additionally constitutes an implemented approach to overcome HSC defects characteristic of many DNA repair deficiency syndromes, like Fanconi anemia.

homology arm (in the 3′ region) to detect specific integration in the proper target locus (9.6 kb) and the unmodified *AAVS1* locus (3.3 kb). To detect the radiolabel signal, auto-radiographic films were used (Amershan Hyperfilm ECL, GE Healthcare) and they were exposed in an automatic reveal machine Curix60 (AGFA).

Supplementary information for this article is available online:
http://embomolmed.embopress.org

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Author contributions
Contribution: PR, RB, AL, LN, and JAB conceived and designed the experiments. PR, RB, AL, OQ-B, LA, ZG, PG, EA, AV, BD, SN, YT, JPT, and RM conducted experiments. JCS, ES, JS, PDG, and MCH provided reagents, tools, and ideas. PR, RB, AL, LN, and JAB wrote the paper.

Conflict of interest
P.D.G. and M.C.H. are current or former employees of Sangamo BioSciences, Inc. The rest of the authors declare that they have no conflict of interest.

For more information
Fanconi Anemia Research Foundation: www.fanconi.org.

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